APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHODS OF DNA SHUFFLING WITH POLYNUCLEOTIDES PRODUCED BY BLOCKING OR INTERRUPTING A SYNTHESIS OR AMPLIFICATION PROCESS

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METHOD OF DNA SHUFFLING WITH POLYNUCLEOTIDES PRODUCED BY BLOCKING OR INTERRUPTING A SYNTHESIS OR AMPLIFICATION PROCESS

Field of the Invention

This invention relates generally to the field of molecular biology and more specifically to the preparation of polynucleotides encoding polypeptides by generating polynucleotides via a procedure involving blocking or interrupting a synthesis or amplification process with an adduct, agent, molecule or other inhibitor, assembling the polynucleotides to form at least one mutant polynucleotide and screening the mutant polynucleotides for the production of a mutant polypeptide(s) having a particular useful property.

Description of the Related Art

An exceedingly large number of possibilities exist for purposeful and random combinations of amino acids within a protein to produce useful mutant proteins and their corresponding biological molecules encoding for the mutant proteins, i.e., DNA, RNA, etc. Accordingly, there is a need to produce and screen a wide variety of such mutant proteins for a useful utility, particularly widely varying random proteins.

The following general discussion of protein and polynucleotide fields may be helpful in further understanding the background for the present invention.

The complexity of an active sequence of a biological macromolecule, e.g., proteins, DNA etc., has been called its information content ("IC"; 5-9), which has been defined as the resistance of the active protein to amino acid sequence variation (calculated from the minimum number of invariable amino acids (bits)) required to describe a family of related sequences with the same function. Proteins that are more sensitive to random mutagenesis have a high information content.

Molecular biology developments such as molecular libraries have allowed the identification of quite a large number of variable bases, and even provide ways to select

functional sequences from random libraries. In such libraries, most residues can be varied (although typically not all at the same time) depending on compensating changes in the context. Thus, while a 100 amino acid protein can contain only 2,000 different mutations, 20^{100} combinations of mutations are possible.

Information density is the Information Content per unit length of a sequence. Active sites of enzymes tend to have a high information density. By contrast, flexible linkers of information in enzymes have a low information density.

Current methods in widespread use for creating mutant proteins in a library format are error-prone polymerase chain reactions and cassette mutagenesis, in which the specific region to be optimized is replaced with a synthetically mutagenized oligonucleotide. In both cases, a cloud of mutant sites is generated around certain sites in the original sequence.

Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. In a mixture of fragments of unknown sequence, error-prone PCR can be used to mutagenize the mixture. The published error-prone PCR protocols suffer from a low processivity of the polymerase. Therefore, the protocol is unable to result in the random mutagenesis of an average-sized gene. This inability limits the practical application of error-prone PCR. Some computer simulations have suggested that point mutagenesis alone may often be too gradual to allow the large-scale block changes that are required for continued and dramatic sequence evolution. Further, the published error-prone PCR protocols do not allow for amplification of DNA fragments greater than 0.5 to 1.0 kb, limiting their practical application. In addition, repeated cycles of error-prone PCR can lead to an accumulation of neutral mutations with undesired results -- such as affecting a protein's immunogenicity but not its binding affinity.

In oligonucleotide-directed mutagenesis, a short sequence is replaced with a synthetically mutagenized oligonucleotide. This approach does not generate combinations of distant mutations and is thus not combinatorial. The limited library size relative to the vast sequence length means that many rounds of selection are unavoidable for protein optimization. Mutagenesis with synthetic oligonucleotides requires sequencing

of individual clones after each selection round followed by grouping them into families, arbitrarily choosing a single family, and reducing it to a consensus motif. Such motif is resynthesized and reinserted into a single gene followed by additional selection. This step process constitutes a statistical bottleneck, is labor intensive, and is not practical for many rounds of mutagenesis.

Error-prone PCR and oligonucleotide-directed mutagenesis are thus useful for single cycles of sequence fine tuning, but rapidly become too limiting when they are applied for multiple cycles.

Another serious limitation of error-prone PCR is that the rate of down-mutations grows with the information content of the sequence. As the information content, library size, and mutagenesis rate increase, the balance of down-mutations to up-mutations will statistically prevent the selection of further improvements (statistical ceiling).

In cassette mutagenesis, a sequence block of a single template is typically replaced by a (partially) randomized sequence. Therefore, the maximum information content that can be obtained is statistically limited by the number of random sequences (i.e., library size). This eliminates other sequence families which are not currently best, but which may have greater long term potential.

Also, mutagenesis with synthetic oligonucleotides requires sequencing of individual clones after each selection round. Thus, such an approach is tedious and impractical for many rounds of mutagenesis.

Thus, error-prone PCR and cassette mutagenesis are best suited, and have been widely used, for fine-tuning areas of comparatively low information content. One apparent exception is the selection of an RNA ligase ribozyme from a random library using many rounds of amplification by error-prone PCR and selection.

It is becoming increasingly clear that the tools for the design of recombinant linear biological sequences such as protein, RNA and DNA are not as powerful as the tools nature has developed. Finding better and better mutants depends on searching more and more sequences within larger and larger libraries, and requiring increased numbers of cycles of mutagenic amplification and selection. However as discussed above, the

existing mutagenesis methods that are in widespread use have distinct limitations when used for repeated cycles.

In nature the evolution of most organisms occurs by natural selection and sexual reproduction. Sexual reproduction ensures mixing and combining of the genes in the offspring of the selected individuals. During meiosis, homologous chromosomes from the parents line up with one another and cross-over part way along their length, thus randomly swapping genetic material. Such swapping or shuffling of the DNA allows organisms to evolve more rapidly.

In sexual recombination, because the inserted sequences were of proven utility in a homologous environment, the inserted sequences are likely to still have substantial information content once they are inserted into the new sequence.

Marton et al. describes the use of PCR *in vitro* to monitor recombination in a plasmid having directly repeated sequences. Marton et al. disclose that recombination will occur during PCR as a result of breaking or nicking of the DNA. This will give rise to recombinant molecules. Meyerhans et al. also disclose the existence of DNA recombination during *in vitro* PCR.

The term Applied Molecular Evolution ("AME") means the application of an evolutionary design algorithm to a specific, useful goal. While many different library formats for AME have been reported for polynucleotides, peptides and proteins (phage, lacI and polysomes), none of these formats have provided for recombination by random cross-overs to deliberately create a combinatorial library.

Theoretically there are 2,000 different single mutants of a 100 amino acid protein. However, a protein of 100 amino acids has 20^{100} possible combinations of mutations, a number which is too large to exhaustively explore by conventional methods. It would be advantageous to develop a system which would allow generation and screening of all of these possible combination mutations.

Some workers in the art have utilized an *in vivo* site specific recombination system to combine light chain antibody genes with heavy chain antibody genes for expression in a phage system. However, their system relies on specific sites of recombination and is limited accordingly. Simultaneous mutagenesis of antibody CDR

regions in single chain antibodies (scFv) by overlapping extension and PCR have been reported.

Others have described a method for generating a large population of multiple mutants using random *in vivo* recombination. However, their method requires the recombination of two different libraries of plasmids, each library having a different selectable marker. Thus, their method is limited to a finite number of recombinations equal to the number of selectable markers existing, and produces a concomitant linear increase in the number of marker genes linked to the selected sequence(s).

In vivo recombination between two homologous but truncated insect-toxin genes on a plasmid have been reported as also being capable of producing a hybrid gene. The *in vivo* recombination of substantially mismatched DNA sequences in a host cell having defective mismatch repair enzymes, resulting in hybrid molecule formation has been reported.

As discussed above, prior methods for producing random proteins from randomized genetic material have met with limited success. Perhaps the best method, thus far, for producing and screening a wide variety of random proteins is a method which utilizes enzymes to cleave (chop) a long nucleotide chain into shorter pieces followed by procedures to separate the chopping agents from the genetic material and procedures to amplify (multiply the copies of) the remaining genetic material in a manner that allows the annealing of the polynucleotides back into chains (either purposefully or randomly put them back together).

A drawback to this method is the expense and inconvenience of utilizing biological enzymes to chop up the genetic material, which are then separated from the genetic material prior to the amplification step. Further, depending upon the particular genetic material, different concentrations of the chopping agents are required to produce the desired fragments. Moreover, the control mechanisms required for biological enzymes are not trivial.

Accordingly, there is a need in the art for producing an improved method of obtaining truly random pieces of genetic material for reassembly to produce random proteins which may be screened for a particular use. The need to produce large libraries

of widely varying mutant nucleic acid sequences is an important goal. Hence, it would be advantageous to develop such a method for the production of mutant proteins which allows for the development of large libraries of mutant nucleic acid sequences which are easily searched. There is a need to develop such a method which allows for the production of large libraries of mutant DNA, RNA or proteins and the selection of particular mutants for a desired goal.

The invention described herein is directed to the use of repeated cycles of mutagenesis, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences, such as DNA, RNA or proteins thorough recombination. It uses repeated cycles of random points mutagenesis, nucleic acid shuffling and selection which allow for the directed molecular evolution *in vitro* of highly complex linear sequences, such as proteins through random recombination.

SUMMARY OF THE INVENTION

The present invention is directed to a method for generating a selected mutant polynucleotide sequence (or a population of selected polynucleotide sequences) typically in the form of amplified and/or cloned polynucleotides, whereby the selected polynucleotide sequences(s) possess at least one desired phenotypic characteristic (e.g., encodes a polypeptide, promotes transcription of linked polynucleotides, binds a protein, and the like) which can be selected for. One method for identifying mutant polypeptides that possess a desired structure or functional property, such as binding to a predetermined biological macromolecule (e.g., a receptor), involves the screening of a large library of polypeptides for individual library members which possess the desired structure or functional property conferred by the amino acid sequence of the polypeptide.

In one embodiment, the present invention provides a method for generating libraries of displayed polypeptides or displayed antibodies suitable for affinity interaction screening or phenotypic screening. The method comprises (1) obtaining a first plurality of selected library members comprising a displayed polypeptide or displayed antibody and an associated polynucleotide encoding said displayed polypeptide or displayed antibody, and obtaining said associated polynucleotides or copies thereof wherein said

associated polynucleotides comprise a region of substantially identical sequences, optimally introducing mutations into said polynucleotides or copies, (2) pooling the polynucleotides or copies, (3) producing smaller or shorter polynucleotides by interrupting a random or particularized priming and synthesis process or an amplification process, and (4) performing amplification, preferably PCR amplification, and optionally mutagenesis to homologously recombine the newly synthesized polynucleotides.

It is a particularly preferred object of the invention to provide a process for producing mutant polynucleotides which express a useful mutant polypeptide by a series of steps comprising:

(a) producing polynucleotides by interrupting a polynucleotide amplification or synthesis process with a means for blocking or interrupting the amplification or synthesis process and thus providing a plurality of smaller or shorter polynucleotides due to the replication of the polynucleotide being in various stages of completion;

(b) adding to the resultant population of single- or double-stranded polynucleotides one or more single- or double-stranded oligonucleotides, wherein said added oligonucleotides comprise an area of identity in an area of heterology to one or more of the single- or double-stranded polynucleotides of the population;

(c) denaturing the resulting single- or double-stranded oligonucleotides to produce a mixture of single-stranded polynucleotides, optionally separating the shorter or smaller polynucleotides into pools of polynucleotides having various lengths and further optionally subjecting said polynucleotides to a PCR procedure to amplify one or more oligonucleotides comprised by at least one of said polynucleotide pools;

(d) incubating a plurality of said polynucleotides or at least one pool of said polynucleotides with a polymerase under conditions which result in annealing of said single-stranded polynucleotides at regions of identity between the single-stranded polynucleotides and thus forming of a mutagenized double-stranded polynucleotide chain;

(e) optionally repeating steps (c) and (d);

(f) expressing at least one mutant polypeptide from said polynucleotide chain, or chains; and

(g) screening said at least one mutant polypeptide for a useful activity.

In a preferred aspect of the invention, the means for blocking or interrupting the amplification or synthesis process is by utilization of uv light, DNA adducts, DNA binding proteins. Preferably, the DNA adduct is a member selected from the group consisting of:

UV light; (+)-CC-1065; (+)-CC-1065-(N3-Adenine); a N-acelylated or deacetylated 4'-fluro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis, or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis; trivalent chromium; a trivalent chromium salt, a polycyclic aromatic hydrocarbon ("PAH") DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[*a*]anthracene ("BMA"); tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"); 2-bromoacrolein (2BA); benzo[*a*]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"); a platinum(II) halogen salt; N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]-quinoline ("N-hydroxy-IQ"); and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*f*]-pyridine ("N-hydroxy-PhIP").

Especially preferred members from the grouping consist of UV light, (+)-CC-1065 and (+)-CC-1065-(N3-Adenine).

In one embodiment of the invention, the DNA adducts, or polynucleotides comprising the DNA adducts, are removed from the polynucleotides or polynucleotide pool, such as by a process including heating the solution comprising the DNA fragments prior to further processing.

Detailed Description of the Invention

The present invention relates to an enhanced method of DNA "shuffling," which may be referred to as "Sexual PCR." In a preferred embodiment of the present invention, amplified or cloned polynucleotides possessing a desired characteristic (for example, encoding a polypeptide of interest, etc.) are selected (via screening of a library of polynucleotides, for example) and pooled. The pooled polynucleotides (or at least one

polynucleotide) may be subjected to random at least one of random primer extension reactions, or PCR amplification using random primers to multiply portions of the polynucleotide or polynucleotides. At various stages along the completion of the PCR amplification or synthesis process, the process may be blocked or interrupted. Hence, a collection of incomplete copies of the polynucleotide or polynucleotides can be generated by random primer extension reactions, amplification using random primers, and/or by pausing or stopping the replication process.

These collections of shorter or smaller polynucleotides (pools) may be isolated or collectively amplified further by PCR, which may be interrupted again. Such "stacking" of the amplification and pausing or stopping steps has the advantage of producing a truly randomized sample of polynucleotides having widely varying lengths. For example, some of the smaller polynucleotides may hybridize with the longer polynucleotides and act as additional random primers to initiate self-priming amplification of polynucleotides within the pool.

Such a process provides an efficient means for producing widely-varying random polynucleotides and subsequent widely-varying mutant proteins corresponding to the same random selection as in the random polynucleotide pool. The reassembly of the shorter or smaller polynucleotides after such shuffling to produce the random polynucleotides may be provided by utilizing procedures standard in the art.

In one embodiment of the invention, the adduct or adducts which halt or slow the PCR process have been modified with a chemical group for which there exists (or can be obtained) a monoclonal antibody specific for the same. Such is an example permitting an efficient separation of polynucleotide chains comprising the DNA adducts (or for the removal of the adducts which have been released from the DNA polynucleotides which comprise them) from other polynucleotide chains. In some situations, it may be desirable to remove such DNA adducts before further processing of the amplified polynucleotides. In other situations it may be desirable to leave such DNA adducts in the solution with the intention of producing a further randomized pool of polynucleotides. Whether the DNA adduct is to be removed or left within the polynucleotide pool depends upon the

composition of the adduct itself and the immediate goal of that amplification process step.

In a preferred embodiment, the polynucleotides produced by interrupting the PCR amplification (and optionally subsequent amplification of the said polynucleotides to produce further randomization under conditions suitable for PCR amplifications) are recombined to form a shuffled pool of recombined polynucleotides, whereby a substantial fraction (e.g., greater than 10 percent) of the recombined polynucleotides of said shuffled pool were not present in the first plurality of selected library members, said shuffled pool providing a library of displayed polypeptides or displayed antibodies suitable for affinity interaction screening.

Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact (e.g., such as catalytic antibodies) with a predetermined macromolecule, such as for example a proteinaceous receptor, peptide oligosaccharide, viron, or other predetermined compound or structure.

The displayed polypeptides, antibodies, peptidomimetic antibodies, and variable region sequences that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (e.g., catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or affinity selection. The method can be modified such that the step of selecting for a phenotypic characteristic can be other than of binding affinity for a predetermined molecule (e.g., for catalytic activity, stability oxidation resistance, drug resistance, or detectable phenotype conferred upon a host cell).

In one embodiment, the first plurality of selected library members is polynucleotides is produced and homologously recombined by PCR *in vitro*, the resultant polynucleotides are transferred into a host cell or organism via a transferring means and homologously recombined to form shuffled library members *in vivo*.

In one embodiment, the first plurality of selected library members is cloned or amplified on episomally replicable vectors, a multiplicity of said vectors is transferred into a cell and homologously recombined to form shuffled library members *in vivo*.

In one embodiment, the first plurality of selected library members is not produced as shorter or smaller polynucleotides, but is cloned or amplified on a episomally replicable vector as a direct repeat, with each repeat comprising a distinct species of selected library member sequence, said vector is transferred into a cell and homologously recombined by intra-vector recombination to form shuffled library members *in vivo*.

In an embodiment, combinations of *in vitro* and *in vivo* shuffling are provided to enhance combinatorial diversity.

The present invention provides a method for generating libraries of displayed antibodies suitable for affinity interactions screening. The method comprises (1) obtaining first a plurality of selected library members comprising a displayed antibody and an associated polynucleotide encoding said displayed antibody, and obtaining said associated polynucleotide encoding for said displayed antibody and obtaining said associated polynucleotides or copies thereof, wherein said associated polynucleotides comprise a region of substantially identical variable region framework sequence, and (2) pooling and producing shorter or smaller polynucleotides with said associated polynucleotides or copies to form polynucleotides under conditions suitable for PCR amplification by slowing or halting the PCR amplification and thereby homologously recombining said shorter or smaller polynucleotides to form a shuffled pool of recombined polynucleotides of said shuffled pool. CDR combinations comprised by the shuffled pool are not present in the first plurality of selected library members, said shuffled pool composing a library of displayed antibodies comprising CDR permutations and suitable for affinity interaction screening. Optionally, the shuffled pool is subjected to affinity screening to select shuffled library members which bind to a predetermined epitope (antigen) and thereby selecting a plurality of selected shuffled library members. Further, the plurality of selectedly shuffled library members can be shuffled and screened iteratively, from 1 to about 1000 cycles or as desired until library members having a desired binding affinity are obtained.

According one aspect of the present invention provides a method for introducing one or more mutations into a template double-stranded polynucleotide,

wherein the template double-stranded polynucleotide has produced polynucleotides of a desired size by the above slowed or halted PCR process, by adding to the resultant population of double stranded polynucleotides one or more single or double stranded oligonucleotides, wherein said oligonucleotides comprise an area of identity and an area of heterology to the template polynucleotide; denaturing the resultant mixture of double-stranded random polynucleotides and oligonucleotides into single-stranded polynucleotides; incubating the resultant population of single-stranded polynucleotides with a polymerase under conditions which result in the annealing of said single-stranded polynucleotides and formation of a mutagenized double-stranded polynucleotide; and repeating the above steps as desired.

In another aspect the present invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under sexual PCR conditions according to the present invention which provide for the production of polynucleotides which include random double-stranded polynucleotides having a desired size and adding to the resultant population of random polynucleotides one or more single or double-stranded oligonucleotides, wherein said oligonucleotides comprise areas of identity and areas of heterology to the template polynucleotide; denaturing the resulting mixture of double-stranded polynucleotides and oligonucleotides into single-stranded polynucleotides; incubating the resultant population of single-stranded polynucleotides with a polymerase under conditions which cause annealing of said single-stranded polynucleotides at the areas of identity to occur and thus to form at least one mutagenized double-stranded polynucleotide; repeating the above steps as desired; and then expressing the recombinant protein from the mutagenized double-stranded polynucleotide.

A third aspect of the present invention is directed to a method for obtaining chimeric polynucleotide by treating a sample comprising different double-stranded template polynucleotides wherein said different template polynucleotides contain areas of identity and areas of heterology under sexual PCR conditions which provide random double-stranded polynucleotides of a desired size from the template polynucleotide;

denaturing the resulting random double-stranded polynucleotides to provide single-stranded polynucleotides; incubating the resulting single-stranded polynucleotides with a polymerase under conditions which provide for the annealing of the single-stranded polynucleotides at the areas of identity and the formation of a chimeric double-stranded polynucleotide sequence comprising template polynucleotide sequences; and repeating the above steps as desired.

A fourth aspect of the present invention is directed to a method of replicating a template polynucleotide by combining *in vitro* single-stranded template polynucleotides with small random single-stranded polynucleotides resulting from the sexual PCR process according to the present invention and denaturation of the template polynucleotide, and incubating said mixture of nucleic acid polynucleotides in the presence of a nucleic acid polymerase under conditions wherein a population of double-stranded template polynucleotides is formed.

The invention also provides the use of polynucleotides shuffling, *in vitro* and/or *in vivo* to shuffle polynucleotides encoding polypeptides and/or polynucleotides comprising transcriptional regulatory sequences.

The invention also provides the use of polynucleotide shuffling to shuffle a population of viral genes (e.g., capsid proteins, spike glycoproteins, polymerases, proteases, etc.) or viral genomes (e.g., paramyxoviridae, orthomyxoviridae, herpesviruses, retroviruses, reoviruses, rhinoviruses, etc.). In an embodiment, the invention provides a method for shuffling sequences encoding all or portions of immunogenic viral proteins to generate novel combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes which are likely to arise in the natural environment as a consequence of viral evolution; (e.g., such as recombination of influenza virus strains).

The invention also provides a method suitable for shuffling polynucleotide sequences for generating gene therapy vectors and replication-defective gene therapy constructs, such as may be used for human gene therapy, including but not limited to

vaccination vectors for DNA-based vaccination, as well as anti-neoplastic gene therapy and other general therapy formats.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a prior art diagram illustrating the resulting mutant polynucleotide from mutations by error-prone PCR as contrasted with those from shuffling and recombination of shorter or smaller polynucleotides.

Figure 2 is a flow chart which illustrates the principles of Sexual PCR in three basic steps: (1) selecting mutants for generation of random sized polynucleotides of polynucleotides, (2) generating random-sized polynucleotides by halting the PCR process, and reassembling the random-sized polynucleotides via PCR to form random polynucleotides.

Figure 3 is a flow chart which illustrates the concepts of utilizing DNA adducts or UV light to halt PCR and to generate random polynucleotides due to random priming and incomplete extension of the ~~strands~~ strands (SEQ ID NOs: 4-9).

Figure 4 is a list of DNA adducts examples and UV light which may be utilized to halt PCR and generate random polynucleotides.

Figure 5 is a flow chart illustrates the steps involved in utilizing UV light to create DNA adducts and halt PCR to generate random ~~polynucleotides~~ polynucleotides (SEQ ID NOs:10-13).

Figures 6A and 6B illustrate the separation of polynucleotides before assembly and the results after assembly, wherein Figure 6A is directed to separation bands of the pre-assembly polynucleotides and Figure 6B is directed in its lane one to illustrating separation bands of reassembled polynucleotides after the first round of reassembly PCR and in lane two illustrating separation bands of reassembled polynucleotides after the second round of reassembly PCR. Lane 2 shows the complete, reassembled random polynucleotide ready for amplification, cloning and screening for a useful utility.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Further advantages of the present invention will become apparent from the following description of the invention with reference to the attached drawings.

The present invention relates to a method for nucleic acid molecule reassembly after producing random oligonucleotides via interrupted PCR, and optionally subjecting at least one of said random oligonucleotides to further PCR as templates to produce additional oligonucleotides, and the application of such reassembly to mutagenesis of DNA sequences. Also described is a method for the production of polynucleotides encoding mutant proteins having enhanced biological activity. In particular, the present invention also relates to a method of utilizing repeated cycles of mutagenesis, nucleic acid shuffling according to the present invention sexual PCR oligonucleotide method and selection which allow for the creation of mutant proteins having enhanced biological activity.

The present invention is directed to a method for generating a very large library of DNA, RNA or protein mutants. This method has particular advantages in the generation of related polynucleotides from which the desired active polynucleotide portion(s) may be selected. In particular the present invention also relates to a method of repeated cycles of mutagenesis, homologous recombination and selection which allow for the creation of mutant proteins having enhanced biological activity.

For clarity and consistency, the following terms will be defined as utilized above, throughout this document and in the claims:

Definitions

The term "DNA reassembly" is used when recombination occurs between identical sequences.

By contrast, the term "DNA shuffling" is used herein to indicate recombination between substantially homologous but non-identical sequences, in some embodiments DNA shuffling may involve crossover via non-homologous recombination, such as via cer/lox and/or flp/frt systems and the like.

The term "amplification" means that the number of copies of a polynucleotide is increased.

The term "identical" or "identity" means that two nucleic acid sequences have the same sequence or a complementary sequence. Thus, "areas of identity" means that regions or areas of a polynucleotide or the overall polynucleotide are identical or complementary to areas of another polynucleotide or the polynucleotide.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous(i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference "TATAC" and is complementary to a reference sequence "GTATA."

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

A "comparison window," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be

compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482 by the homology alignment algorithm of Needlemen and Wuncsch J. Mol. Biol. 48: 443 (1970), by the search of similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. This "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence having at least 80 percent sequence identity, preferably at least 85 percent identity, often 90 to 95 percent sequence identity, and most commonly at least 99 percent sequence identity as compared to a reference sequence of a comparison window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

"Conservative amino acid substitutions" refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are : valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "homologous" or "homeologous" means that one single-stranded nucleic acid nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentrations as discussed later. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

The term "heterologous" means that one single-stranded nucleic acid sequence is unable to hybridize to another single-stranded nucleic acid sequence or its complement. Thus areas of heterology means that areas of polynucleotides or polynucleotides have areas or regions within their sequence which are unable to hybridize to another nucleic acid or polynucleotide. Such regions or areas are, for example areas of mutations.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome the human CD4 gene is the cognate gene to the mouse 3d4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition.

The term "wild-type" means that the polynucleotide does not comprise any mutations. A "wild type" protein means that the protein will be active at a level of activity found in nature and will comprise the amino acid sequence found in nature.

The term "related polynucleotides" means that regions or areas of the polynucleotides are identical and regions or areas of the polynucleotides are heterologous.

The term "chimeric polynucleotide" means that the polynucleotide comprises regions which are wild -type and regions which are mutated. It may also meant the polynucleotide comprises wild-type regions form one polynucleotide and wild-type regions form another related polynucleotide.

The term "cleaving" means digesting the polynucleotide with enzymes or breaking the polynucleotide.

The term "population" as used herein means a collection of components such as polynucleotides, portions or polynucleotides or proteins. A "mixed population: means a collection of components which belong to the same family of nucleic acids or proteins (i.e., are related) but which differ in their sequence (i.e., are not identical) and hence in their biological activity.

The term "specific polynucleotide" means a polynucleotide having certain end points and having a certain nucleic acid sequence. Two polynucleotides wherein one polynucleotide has the identical sequence as a portion of the second polynucleotide but different ends comprises two different specific polynucleotides.

The term "mutations" means changes in the sequence of a wild-type nucleic acid sequence or changes in the sequence of a peptide. Such mutations may be pint mutations such as transitions or transversions. The mutations may be deletions, insertions or duplications.

In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction.

The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences".

The term "naturally-occurring" as used herein as applied to the object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring. Generally, the term naturally occurring refers to an object as present in a non-pathological (un-diseased) individual, such as would be typical for the species.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds (e.g., a VLSIPS peptide array, polynucleotide array, and/or combinatorial small molecule array), biological macromolecule, a bacteriophage peptide display library, a bacteriophage antibody (e.g., scFv) display library, a polysome peptide display library, or an extract made form biological materials such as bacteria, plants, fungi, or animal (particular mammalian) cells or tissues. Agents are evaluated for potential activity as anti-neoplastics, anti-inflammatories or apoptosis modulators by inclusion in screening assays described hereinbelow. Agents are evaluated for potential activity as specific protein interaction inhibitors (i.e., an agent which selectively inhibits a binding interaction between two predetermined polypeptides but which doe snot substantially interfere with cell viability) by inclusion in screening assays described hereinbelow.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual macromolecular species in the composition), and preferably substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a

substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species.

As used herein the term "physiological conditions" refers to temperature, pH, ionic strength, viscosity, and like biochemical parameters which are compatible with a viable organism, and/or which typically exist intracellularly in a viable cultured yeast cell or mammalian cell. For example, the intracellular conditions in a yeast cell grown under typical laboratory culture conditions are physiological conditions. Suitable *in vitro* reaction conditions for *in vitro* transcription cocktails are generally physiological conditions. In general, *in vitro* physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45°C and 0.001-10 mM divalent cation (e.g., Mg^{++}, Ca^{++}); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (e.g., BSA). A non-ionic detergent (~~Tween,~~ TWEEN® detergent NP-40, ~~Triton~~ TRITON® detergent X-100) can often be present, usually at about 0.001 to 2%, typically 0.05-0.2% (v/v). Particular aqueous conditions may be selected by the practitioner according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or non-ionic detergents and/or membrane fractions and/or anti-foam agents and/or scintillants.

"Specific hybridization" is defined herein as the formation of hybrids between a first polynucleotide and a second polynucleotide (e.g., a polynucleotide having a distinct but substantially identical sequence to the first polynucleotide), wherein substantially unrelated polynucleotide sequences do not form hybrids in the mixture.

As used herein, the term "single-chain antibody" refers to a polypeptide comprising a V_H domain and a V_L domain in polypeptide linkage, generally liked via a spacer peptide (e.g., ~~[Gly-Gly-Gly-Gly-Ser]$_x$~~ [Gly-Gly-Gly-Gly-Ser]$_x$ (SEQ ID NO:1)), and which may comprise additional amino acid sequences at the amino- and/or carboxy- termini. For example, a single-chain

antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a scFv is a single-chain antibody. Single-chain antibodies are generally proteins consisting of one or more polypeptide segments of at least 10 contiguous amino substantially encoded by genes of the immunoglobulin superfamily (e.g., see The Immunoglobulin Gene Superfamily, A.F. Williams and A.N. Barclay, in Immunoglobulin Genes, T. Honjo, F.W. Alt, and THE. Rabbits, eds., (1989) Academic press: San Diego, CA, pp. 361-368, which is incorporated herein by reference), most frequently encoded by a rodent, non-human primate, avian, porcine bovine, ovine, goat, or human heavy chain or light chain gene sequence. A functional single-chain antibody generally contains a sufficient portion of an immunoglobulin superfamily gene product so as to retain the property of binding to a specific target molecule, typically a receptor or antigen (epitope).

As used herein, the term "complementarity-determining region" and "CDR" refer to the art-recognized term as exemplified by the Kabat and Chothia CDR definitions also generally known as supervariable regions or hypervariable loops (Chothia and Leks (1987) J. Mol. Biol. 196; 901; Clothia et al. (1989) Nature 342; 877; E.A. Kabat et al., Sequences of Proteins of Immunological Interest (national Institutes of Health, Bethesda, MD) (1987); and Tramontano et al. (1990) J. Mol. Biolog. 215; 175). Variable region domains typically comprise the amino-terminal approximately 105-115 amino acids of a naturally-occurring immunoglobulin chain (e.g., amino acids 1-110), although variable domains somewhat shorter or longer are also suitable for forming single-chain antibodies.

An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, also called CDR's. The extent of the framework region and CDR's have been precisely defined (see, "Sequences of Proteins of Immunological Interest," E. Kabat et al., 4th Ed., U.S. Department of Health and human services, Bethesda, MD (1987)). The sequences of the framework regions of different light or heavy chains are relatively conserved within a specie. As used herein, a "human framework region" is a framework region that is substantially identical (about 85 or more, usually 90-95 or more) to the framework region of a

naturally occurring human immunoglobulin. the framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDR's. The CDR's are primarily responsible for binding to an epitope of an antigen.

As used herein, the term "variable segment" refers to a portion of a nascent peptide which comprises a random, pseudorandom, or defined kernal sequence. A variable segment" refers to a portion of a nascent peptide which comprises a random pseudorandom, or defined kernal sequence. A variable segment can comprise both variant and invariant residue positions, and the degree of residue variation at a variant residue position may be limited: both options are selected at the discretion of the practitioner. Typically, variable segments are about 5 to 20 amino acid residues in length (e.g., 8 to 10), although variable segments may be longer and may comprise antibody portions or receptor proteins, such as an antibody fragment, a nucleic acid binding protein, a receptor protein, and the like.

As used herein, "random peptide sequence" refers to an amino acid sequence composed of two or more amino acid monomers and constructed by a stochastic or random process. A random peptide can include framework or scaffolding motifs, which may comprise invariant sequences.

As used herein "random peptide library" refers to a set of polynucleotide sequences that encodes a set of random peptides, and to the set of random peptides encoded by those polynucleotide sequences, as well as the fusion proteins contain those random peptides.

As used herein, the term "pseudorandom" refers to a set of sequences that have limited variability, sot that for example the degree of residue variability at another position, but any pseudorandom position is allowed some degree of residue variation, however circumscribed.

As used herein, the term "defined sequence framework" refers to a set of defined sequences that are selected on a non-random basis, generally on the basis of experimental data or structural data; for example, a defined sequence framework may comprise a set of amino acid sequences that are predicted to form a ß-sheet structure or

may comprise a leucine zipper heptad repeat motif, a zinc-finger domain, among other variations. A "defined sequence kernal" is a set of sequences which encompass a limited scope of variability. Whereas (1) a completely random 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences, and (2) a pseudorandom 10-mer sequence of the 20 conventional amino acids can be any of $(20)^{10}$ sequences but will exhibit a bias for certain residues at certain positions and/or overall, (3) a defined sequence kernal is a subset of sequences if each residue position was allowed to be any of the allowable 20 conventional amino acids (and/or allowable unconventional amino/imino acids). A defined sequence kernal generally comprises variant and invariant residue positions and/or comprises variant residue positions which can comprise a residue selected from a defined subset of amino acid residues), and the like, either segmentally or over the entire length of the individual selected library member sequence. Defined sequence kernels can refer to either amino acid sequences or polynucleotide sequences. Of illustration and not limitation, the sequences ~~(NNK)$_{10}$~~ $(NNK)_{10}$ (SEQ ID NO:2) and ~~(NNM)$_{10}$~~ $(NNM)_{10}$ (SEQ ID NO:3), wherein N represents A, T, G, or C; K represents G or T; and M represents A or C, are defined sequence kernels.

As used herein "epitope" refers to that portion of an antigen or other macromolecule capable of forming a binding interaction that interacts with the variable region binding body of an antibody. Typically, such binding interaction is manifested as an intermolecular contact with one or more amino acid residues of a CDR.

As used herein, "receptor" refers to a molecule that has an affinity for a given ligand. Receptors can be naturally occurring or synthetic molecules. Receptors can be employed in an unaltered state or as aggregates with other species. Receptors can be attached, covalently or non-covalently, to a binding member, either directly or via a specific binding substance. Examples of receptors include, but are not limited to, antibodies, including monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), cell membrane receptors, complex carbohydrates and glycoproteins, enzymes, and hormone receptors.

As used herein "ligand" refers to a molecule, such as a random peptide or variable segment sequence, that is recognized by a particular receptor. As one of skill

in the art will recognize, a molecule (or macromolecular complex) can be both a receptor and a ligand. In general, the binding partner having a smaller molecular weight is referred to as the ligand and the binding partner having a greater molecular weight is referred to as a receptor.

As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two molecules, such as a DNA binding protein and a random peptide, and serves to place the two molecules in a preferred configuration, e.g., so that the random peptide can bind to a receptor with minimal steric hindrance from the DNA binding protein.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

As used herein, the "means for slowing or halting the PCR amplification process" is defined as utilization of UV light or a DNA adduct to slow or halt the PCR amplification of at least one polynucleotide. Preferably, such a means is either UV light or a DNA adduct which is a member selected from the group consisting of: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine), (see. Biochem. 31, 2822-2829 (1992)); a N-acelylated or deacetylated 4'-fluro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see, for example, Carcinogenesis vol. 13, No. 5, 751-758 (1992); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see also, Id.. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon ("PAH") DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[*a*]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[*a*]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]-quinoline ("N-

hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*f*]-pyridine ("N-hydroxy-PhIP"). Especially preferred "means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotides pool, which can be released or removed by a process including heating the solution comprising the polynucleotides prior to further processing.

Methodology

Nucleic acid shuffling is a method for *in vitro* or *in vivo* homologous recombination of pools of shorter or smaller polynucleotides to produce a polynucleotide or polynucleotides. Mixtures of related nucleic acid sequences or polynucleotides are subjected to sexual PCR to provide random polynucleotides, and reassembled to yield a library or mixed population of recombinant mutant nucleic acid molecules or polynucleotides.

In contrast to cassette mutagenesis, only shuffling and error-prone PCR allow one to mutate a pool of sequences blindly (without sequence information other than primers).

The advantage of the mutagenic shuffling of this invention over error-prone PCR alone for repeated selection can best be explained with an example from antibody engineering. In Figure 1 is shown a prior art schematic diagram of DNA shuffling as compared with error-prone PCR (not sexual PCR). The initial library of selected pooled sequences can consist of related sequences of diverse origin (i.e. antibodies from naive mRNA) or can be derived by any type of mutagenesis (including shuffling) of a single antibody gene. A collection of selected complementarity determining regions ("CDRs") is obtained after the first round of affinity selection (Fig. 1). In the diagram the thick CDRs confer onto the antibody molecule increased affinity for the antigen. Shuffling allows the free combinatorial association of all of the CDR1s with all of the CDR2s with all of the CDR3s, etc.

This method differs from error-prone PCR, in that it is an inverse chain reaction. In error-prone PCR, the number of polymerase start sites and the number of molecules grows exponentially. However, the sequence of the polymerase start sites and the sequence of the molecules remains essentially the same. In contrast, in nucleic acid reassembly or shuffling of random polynucleotides the number of start sites and the number (but not size) of the random polynucleotides decreases over time. For polynucleotides derived from whole plasmids the theoretical endpoint is a single, large concatemeric molecule.

Since cross-overs occur at regions of homology, recombination will primarily occur between members of the same sequence family. This discourages combinations of CDRs that are grossly incompatible (e.g., directed against different epitopes of the same antigen). It is contemplated that multiple families of sequences can be shuffled in the same reaction. Further, shuffling generally conserves the relative order, such that, for example, CDR1 will not be found in the position of CDR2.

Rare shufflants will contain a large number of the best (eg. highest affinity) CDRs and these rare shufflants may be selected based on their superior affinity (Fig. 1). CDRs from a pool of 100 different selected antibody sequences can be permutated in up to 1006 different ways. This large number of permutations cannot be represented in a single library of DNA sequences. Accordingly, it is contemplated that multiple cycles of DNA shuffling and selection may be required depending on the length of the sequence and the sequence diversity desired.

Error-prone PCR, in contrast, keeps all the selected CDRs in the same relative sequence (Fig. 1), generating a much smaller mutant cloud.

The template polynucleotide which may be used in the methods of this invention may be DNA or RNA. It may be of various lengths depending on the size of the gene or shorter or smaller polynucleotide to be recombined or reassembled. Preferably, the template polynucleotide is from 50 bp to 50 kb. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest can be used in the methods of this invention, and in fact have been successfully used.

The template polynucleotide may be obtained by amplification using the PCR reaction (U.S. Patent No. 4,683,202 and 4,683,195) or other amplification or cloning methods. However, the removal of free primers from the PCR products before subjecting them to pooling of the PCR products and sexual PCR may provide more efficient results. Failure to adequately remove the primers from the original pool before sexual PCR can lead to a low frequency of crossover clones.

The template polynucleotide often should be double-stranded. A double-stranded nucleic acid molecule is recommended to ensure that regions of the resulting single-stranded polynucleotides are complementary to each other and thus can hybridize to form a double-stranded molecule.

It is contemplated that single-stranded or double-stranded nucleic acid polynucleotides having regions of identity to the template polynucleotide and regions of heterology to the template polynucleotide may be added to the template polynucleotide, at this step. It is also contemplated that two different but related polynucleotide templates can be mixed at this step.

The double-stranded polynucleotide template and any added double-or single-stranded polynucleotides are subjected to sexual PCR which includes slowing or halting to provide a mixture of from about 5 bp to 5 kb or more. Preferably the size of the random polynucleotides is from about 10 bp to 1000 bp, more preferably the size of the polynucleotides is from about 20 bp to 500 bp.

Alternatively, it is also contemplated that double-stranded nucleic acid having multiple nicks may be used in the methods of this invention. A nick is a break in one strand of the double-stranded nucleic acid. The distance between such nicks is preferably 5 bp to 5 kb, more preferably between 10 bp to 1000 bp. This can provide areas of self-priming to produce shorter or smaller polynucleotides to be included with the polynucleotides resulting from random primers, for example.

The concentration of any one specific polynucleotide will not be greater than 1% by weight of the total polynucleotides, more preferably the concentration of any one specific nucleic acid sequence will not be greater than 0.1% by weight of the total nucleic acid.

The number of different specific polynucletides in the mixture will be at least about 100, preferably at least about 500, and more preferably at least about 1000.

At this step single-stranded or double-stranded polynucleotides, either synthetic or natural, may be added to the random double-stranded shorter or smaller polynucleotides in order to increase the heterogeneity of the mixture of polynucleotides.

It is also contemplated that populations of double-stranded randomly broken polynucleotides may be mixed or combined at this step with the polynucleotides from the sexual PCR process and optionally subjected to one or more additional sexual PCR cycles.

Where insertion of mutations into the template polynucleotide is desired, single-stranded or double-stranded polynucleotides having a region of identity to the template polynucleotide and a region of heterology to the template polynucleotide may be added in a 20 fold excess by weight as compared to the total nucleic acid, more preferably the single-stranded polynucleotides may be added in a 10 fold excess by weight as compared to the total nucleic acid.

Where a mixture of different but related template polynucleotides is desired, populations of polynucleotides from each of the templates may be combined at a ratio of less than about 1:100, more preferably the ratio is less than about 1:40. For example, a backcross of the wild-type polynucleotide with a population of mutated polynucleotide may be desired to eliminate neutral mutations (e.g., mutations yielding an insubstantial alteration in the phenotypic property being selected for). In such an example, the ratio of randomly provided wild-type polynucleotides which may be added to the randomly provided sexual PCR cycle mutant polynucleotides is approximately 1:1 to about 100:1, and more preferably from 1:1 to 40:1.

The mixed population of random polynucleotides are denatured to form single-stranded polynucleotides and then re-annealed. Only those single-stranded polynucleotides having regions of homology with other single-stranded polynucleotides will re-anneal.

The random polynucleotides may be denatured by heating. One skilled in the art could determine the conditions necessary to completely denature the double-stranded

nucleic acid. Preferably the temperature is from 80 °C to 100 °C, more preferably the temperature is from 90 °C to 96 °C. other methods which may be used to denature the polynucleotides include pressure (36) and pH.

The polynucleotides may be re-annealed by cooling. Preferably the temperature is from 20 °C to 75 °C, more preferably the temperature is from 40 °C to 65 °C. If a high frequency of crossovers is needed based on an average of only 4 consecutive bases of homology, recombination can be forced by using a low annealing temperature, although the process becomes more difficult. The degree of renaturation which occurs will depend on the degree of homology between the population of single-stranded polynucleotides.

Renaturation can be accelerated by the addition of polyethylene glycol ("PEG") or salt. The salt concentration is preferably from 0 mM to 200 mM, more preferably the salt concentration is from 10 mM to 100 mm. The salt may be KCl or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%.

The annealed polynucleotides are next incubated in the presence of a nucleic acid polymerase and dNTP's (i.e. DATP, dCTP, DGTP and DTTP). The nucleic acid polymerase may be the Klenow fragment, the ~~Taq~~ TAQ® polymerase or any other DNA polymerase known in the art.

The approach to be used for the assembly depends on the minimum degree of homology that should still yield crossovers. If the areas of identity are large, ~~Taq~~ TAQ® polymerase can be used with an annealing temperature of between 45-65 °C. If the areas of identity are small, Klenow polymerase can be used with an annealing temperature of between 20-30 °C. One skilled in the art could vary the temperature of annealing to increase the number of cross-overs achieved.

The polymerase may be added to the random polynucleotides prior to annealing, simultaneously with annealing or after annealing.

The cycle of denaturation, renaturation and incubation in the presence of polymerase is referred to herein as shuffling or reassembly of the nucleic acid. This

cycle is repeated for a desired number of times. Preferably the cycle is repeated from 2 to 50 times, more preferably the sequence is repeated from 10 to 40 times.

The resulting nucleic acid is a larger double-stranded polynucleotide of from about 50 bp to about 100 kb, preferably the larger polynucleotide is from 500 bp to 50 kb.

This larger polynucleotides may contain a number of copies of a polynucleotide having the same size as the template polynucleotide in tandem. This concatemeric polynucleotide is then denatured into single copies of the template polynucleotide. The result will be a population of polynucleotides of approximately the same size as the template polynucleotide. The population will be a mixed population where single or double-stranded polynucleotides having an area of identity and an area of heterology have been added to the template polynucleotide prior to shuffling.

These polynucleotides are then cloned into the appropriate vector and the ligation mixture used to transform bacteria.

It is contemplated that the single polynucleotides may be obtained from the larger concatemeric polynucleotide by amplification of the single polynucleotide prior to cloning by a variety of methods including PCR (U.S. Patent No. 4,683,195 and 4,683,202), rather than by digestion of the concatemer.

The vector used for cloning is not critical provided that it will accept a polynucleotide of the desired size. If expression of the particular polynucleotide is desired, the cloning vehicle should further comprise transcription and translation signals next to the site of insertion of the polynucleotide to allow expression of the polynucleotide in the host cell. Preferred vectors include the pUC series and the pBR series of plasmids.

The resulting bacterial population will include a number of recombinant polynucleotides having random mutations. This mixed population may be tested to identify the desired recombinant polynucleotides. The method of selection will depend on the polynucleotide desired.

For example, if a polynucleotide which encodes for a protein with increased binding efficiency to a ligand is desired, the proteins expressed by each of the portions

of the polynucleotides in the population or library may be tested for their ability to bind to the ligand by methods known in the art (i.e. panning, affinity chromatography). If a polynucleotide which encodes for a protein with increased drug resistance is desired, the proteins expressed by each of the polynucleotides in the population or library may be tested for their ability to confer drug resistance to the host organism. One skilled in the art, given knowledge of the desired protein, could readily test the population to identify polynucleotides which confer the desired properties onto the protein.

It is contemplated that one skilled in the art could use a phage display system in which fragments of the protein are expressed as fusion proteins on the phage surface (Pharmacia, Milwaukee WI). The recombinant DNA molecules are cloned into the phage DNA at a site which results in the transcription of a fusion protein a portion of which is encoded by the recombinant DNA molecule. The phage containing the recombinant nucleic acid molecule undergoes replication and transcription in the cell. The leader sequence of the fusion protein directs the transport of the fusion protein to the tip of the phage particle. Thus the fusion protein which is partially encoded by the recombinant DNA molecule is displayed on the phage particle for detection and selection by the methods described above.

It is further contemplated that a number of cycles of nucleic acid shuffling may be conducted with polynucleotides from a sub-population of the first population, which sub-population contains DNA encoding the desired recombinant protein. In this manner, proteins with even higher binding affinities or enzymatic activity could be achieved.

It is also contemplated that a number of cycles of nucleic acid shuffling may be conducted with a mixture of wild-type polynucleotides and a sub-population of nucleic acid from the first or subsequent rounds of nucleic acid shuffling in order to remove any silent mutations from the sub-population.

Any source of nucleic acid, in purified form can be utilized as the starting nucleic acid. Thus the process may employ DNA or RNA including messenger RNA, which DNA or RNA may be single or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. The nucleic acid sequence

may be of various lengths depending on the size of the nucleic acid sequence to be mutated. Preferably the specific nucleic acid sequence is from 50 to 50000 base pairs. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest may be used in the methods of this invention.

The nucleic acid may be obtained from any source, for example, from plasmids such a pBR322, from cloned DNA or RNA or from natural DNA or RNA from any source including bacteria, yeast, viruses and higher organisms such as plants or animals. DNA or RNA may be extracted from blood or tissue material. The template polynucleotide may be obtained by amplification using the polynucleotide chain reaction (PCR) (U.S. Patent no. 4,683,202 and 4,683,195). Alternatively, the polynucleotide may be present in a vector present in a cell and sufficient nucleic acid may be obtained by culturing the cell and extracting the nucleic acid from the cell by methods known in the art.

Any specific nucleic acid sequence can be used to produce the population of mutants by the present process. It is only necessary that a small population of mutant sequences of the specific nucleic acid sequence exist or be created prior to the present process.

The initial small population of the specific nucleic acid sequences having mutations may be created by a number of different methods. Mutations may be created by error-prone PCR. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. Alternatively, mutations can be introduced into the template polynucleotide by oligonucleotide-directed mutagenesis. In oligonucleotide-directed mutagenesis, a short sequence of the polynucleotide is removed from the polynucleotide using restriction enzyme digestion and is replaced with a synthetic polynucleotide in which various bases have been altered from the original sequence. The polynucleotide sequence can also be altered by chemical mutagenesis. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Generally, these agents are added to the PCR reaction in place of the nucleotide

precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid polynucleotides so mutagenized are introduced into *E. coli* and propagated as a pool or library of mutant plasmids.

Alternatively the small mixed population of specific nucleic acids may be found in nature in that they may consist of different alleles of the same gene or the same gene from different related species (i.e., cognate genes). Alternatively, they may be related DNA sequences found within one species, for example, the immunoglobulin genes.

Once the mixed population of the specific nucleic acid sequences is generated, the polynucleotides can be used directly or inserted into an appropriate cloning vector, using techniques well-known in,the art.

The choice of vector depends on the size of the polynucleotide sequence and the host cell to be employed in the methods of this invention. The templates of this invention may be plasmids, phages, cosmids, phagemids, viruses (e.g., retroviruses, parainfluenzavirus, herpesviruses, reoviruses, paramyxoviruses, and the like), or selected portions thereof (e. g., coat protein, spike glycoprotein, capsid protein). For example, cosmids and phagemids are preferred where the specific nucleic acid sequence to be mutated is larger because these vectors are able to stably propagate large polynucleotides.

If the mixed population of the specific nucleic acid sequence is cloned into a vector it can be clonally amplified by inserting each vector into a host cell and allowing the host cell to amplify the vector. This is referred to as clonal amplification because while the absolute number of nucleic acid sequences increases, the number of mutants does not increase. Utility can be readily determined by screening expressed polypeptides.

The DNA shuffling method of this invention can be performed blindly on a pool of unknown sequences. By adding to the reassembly mixture oligonucleotides (with ends that are homologous to the sequences being reassembled) any sequence mixture can

be incorporated at any specific position into another sequence mixture. Thus, it is contemplated that mixtures of synthetic oligonucleotides, PCR polynucleotides or even whole genes can be mixed into another sequence library at defined positions. The insertion of one sequence (mixture) is independent from the insertion of a sequence in another part of the template. Thus, the degree of recombination, the homology required, and the diversity of the library can be independently and simultaneously varied along the length of the reassembled DNA.

This approach of mixing two genes may be useful for the humanization of antibodies from murine hybridomas. The approach of mixing two genes or inserting mutant sequences into genes may be useful for any therapeutically used protein, for example, interleukin I, antibodies, tPA, growth hormone, etc. The approach may also be useful in any nucleic acid for example, promoters or introns or 31 untranslated region or 51 untranslated regions of genes to increase expression or alter specificity of expression of proteins. The approach may also be used to mutate ribozymes or aptamers.

Shuffling requires the presence of homologous regions separating regions of diversity. Scaffold-like protein structures may be particularly suitable for shuffling. The conserved scaffold determines the overall folding by self-association, while displaying relatively unrestricted loops that mediate the specific binding. Examples of such scaffolds are the immunoglobulin beta-barrel, and the four-helix bundle which are well-known in the art. This shuffling can be used to create scaffold-like proteins with various combinations of mutated sequences for binding.

<u>*In Vitro* Shuffling</u>

The equivalents of some standard genetic matings may also be performed by shuffling *in vitro*. For example, a "molecular backcross" can be performed by repeatedly mixing the mutant's nucleic acid with the wild-type nucleic acid while selecting for the mutations of interest. As in traditional breeding, this approach can be used to combine phenotypes from different sources into a background of choice. It is useful, for example, for the removal of neutral mutations that affect unselected characteristics (i.e. immunogenicity). Thus it can be useful to determine which mutations in a protein are

involved in the enhanced biological activity and which are not, an advantage which cannot be achieved by error-prone mutagenesis or cassette mutagenesis methods.

Large, functional genes can be assembled correctly from a mixture of small random polynucleotides. This reaction may be of use for the reassembly of genes from the highly fragmented DNA of fossils. In addition random nucleic acid fragments from fossils may be combined with polynucleotides from similar genes from related species.

It is also contemplated that the method of this invention can be used for the *in vitro* amplification of a whole genome from a single cell as is needed for a variety of research and diagnostic applications. DNA amplification by PCR is in practice limited to a length of about 40 kb. Amplification of a whole genome such as that of *E.* coli (5, 000 kb) by PCR would require about 250 primers yielding 125 forty kb polynucleotides. This approach is not practical due to the unavailability of sufficient sequence data. On the other hand, random production of polynucleotides of the genome with sexual PCR cycles, followed by gel purification of small polynucleotides will provide a multitude of possible primers. Use of this mix of random small polynucleotides as primers in a PCR reaction alone or with the whole genome as the template should result in an inverse chain reaction with the theoretical endpoint of a single concatemer containing many copies of the genome.

100 fold amplification in the copy number and an average polynucleotide size of greater than 50 kb may be obtained when only random polynucleotides are used. It is thought that the larger concatemer is generated by overlap of many smaller polynucleotides. The quality of specific PCR products obtained using synthetic primers will be indistinguishable from the product obtained from unamplified DNA. It is expected that this approach will be useful for the mapping of genomes.

The polynucleotide to be shuffled can be produced as random or non-random polynucleotides, at the discretion of the practitioner.

In Vivo Shuffling

In an embodiment of *in vivo* shuffling, the mixed population of the specific nucleic acid sequence is introduced into bacterial or eukaryotic cells under conditions

such that at least two different nucleic acid sequences are present in each host cell. The polynucleotides can be introduced into the host cells by a variety of different methods. The host cells can be transformed with the smaller polynucleotides using methods known in the art, for example treatment with calcium chloride. If the polynucleotides are inserted into a phage genome, the host cell can be transfected with the recombinant phage genome having the specific nucleic acid sequences. Alternatively, the nucleic acid sequences can be introduced into the host cell using electroporation, transfection, lipofection, biolistics, conjugation, and the like.

In general, in this embodiment, the specific nucleic acids sequences will be present in vectors which are capable of stably replicating the sequence in the host cell. In addition, it is contemplated that the vectors will encode a marker gene such that host cells having the vector can be selected. This ensures that the mutated specific nucleic acid sequence can be recovered after introduction into the host cell. However, it is contemplated that the entire mixed population of the specific nucleic acid sequences need not be present on a vector sequence. Rather only a sufficient number of sequences need be cloned into vectors to ensure that after introduction of the polynucleotides into the host cells each host cell contains one vector having at least one specific nucleic acid sequence present therein. It is also contemplated that rather than having a subset of the population of the specific nucleic acids sequences cloned into vectors, this subset may be already stably integrated into the host cell.

It has been found that when two polynucleotides which have regions of identity are inserted into the host cells homologous recombination occurs between the two polynucleotides. Such recombination between the two mutated specific nucleic acid sequences will result in the production of double or triple mutants in some situations.

It has also been found that the frequency of recombination is increased if some of the mutated specific nucleic acid sequences are present on linear nucleic acid molecules. Therefore, in a preferred embodiment, some of the specific nucleic acid sequences are present on linear polynucleotides.

After transformation, the host cell transformants are placed under selection to identify those host cell transformants which contain mutated specific nucleic acid

sequences having the qualities desired. For example, if increased resistance to a particular drug is desired then the transformed host cells may be subjected to increased concentrations of the particular drug and those transformants producing mutated proteins able to confer increased drug resistance will be selected. If the enhanced ability of a particular protein to bind to a receptor is desired, then expression of the protein can be induced from the transformants and the resulting protein assayed in a ligand binding assay by methods known in the art to identify that subset of the mutated population which shows enhanced binding to the ligand. Alternatively, the protein can be expressed in another system to ensure proper processing.

Once a subset of the first recombined specific nucleic acid sequences (daughter sequences) having the desired characteristics are identified, they are then subject to a second round of recombination.

In the second cycle of recombination, the recombined specific nucleic acid sequences may be mixed with the original mutated specific nucleic acid sequences (parent sequences) and the cycle repeated as described above. In this way a set of second recombined specific nucleic acids sequences can be identified which have enhanced characteristics or encode for proteins having enhanced properties. This cycle can be repeated a number of times as desired.

It is also contemplated that in the second or subsequent recombination cycle, a backcross can be performed. A molecular backcross can be performed by mixing the desired specific nucleic acid sequences with a large number of the wild-type sequence, such that at least one wild-type nucleic acid sequence and a mutated nucleic acid sequence are present in the same host cell after transformation. Recombination with the wild-type specific nucleic acid sequence will eliminate those neutral mutations that may affect unselected characteristics such as immunogenicity but not the selected characteristics.

In another embodiment of this invention, it is contemplated that during the first round a subset of the specific nucleic acid sequences can be generated as smaller polynucleotides by slowing or halting their PCR amplification prior to introduction into the host cell. The size of the polynucleotides must be large enough to contain some

regions of identity with the other sequences so as to homologously recombine with the other sequences. The size of the polynucleotides will range from 0.03 kb to 100 kb more preferably from 0. 2 kb to 10 kb. It is also contemplated that in subsequent rounds, all of the specific nucleic acid sequences other than the sequences selected from the previous round may be utilized to generate PCR polynucleotides prior to introduction into the host cells.

The shorter polynucleotide sequences can be single-stranded or double-stranded. If the sequences were originally single-stranded and have become double-stranded they can be denatured with heat, chemicals or enzymes prior to insertion into the host cell. The reaction conditions suitable for separating the strands of nucleic acid are well known in the art.

The steps of this process can be repeated indefinitely, being limited only by the number of possible mutants which dan be achieved. After a certain number of cycles, all possible mutants will have been achieved and further cycles are redundant.

In an embodiment the same mutated template nucleic acid is repeatedly recombined and the resulting recombinants selected for the desired characteristic.

Therefore, the initial pool or population of mutated template nucleic acid is cloned into a vector capable of replicating in a bacteria such as *E. coli.* The particular vector is not essential, so long as it is capable of autonomous replication in E. coli. In a preferred embodiment, the vector is designed to allow the expression and production of any protein encoded by the mutated specific nucleic acid linked to the vector. It is also preferred that the vector contain a gene encoding for a selectable marker.

The population of vectors containing the pool of mutated nucleic acid sequences is introduced into the E. coli host cells. The vector nucleic acid sequences may be introduced by transformation, transfection or infection in the case of phage. The concentration of vectors used to transform the bacteria is such that a number of vectors is introduced into each cell. Once present in the cell, the efficiency of homologous recombination is such that homologous recombination occurs between the various

vectors. This results in the generation of mutants (daughters) having a combination of mutations which differ from the original parent mutated sequences.

The host cells are then clonally replicated and selected for the marker gene present on the vector. Only those cells having a plasmid will grow under the selection.

The host cells which contain a vector are then tested for the presence of favorable mutations. Such testing may consist of placing the cells under selective pressure, for example, if the gene to be selected is an improved drug resistance gene. If the vector allows expression of the protein encoded by the mutated nucleic acid sequence, then such selection may include allowing expression of the protein so encoded, isolation of the protein and testing of the protein to determine whether, for example, it binds with increased efficiency to the ligand of interest.

Once a particular daughter mutated nucleic acid sequence has been identified which confrs the desired characteristics, the nucleic acid is isolated either already linked to the vector or separated from the vector. This nucleic acid is then mixed with the first or parent population of nucleic acids and the cycle is repeated.

It has been shown that by this method nucleic acid sequences having enhanced desired properties can be selected.

In an alternate embodiment, the first generation of mutants are retained in the cells and the parental mutated sequences are added again to the cells. Accordingly, the first cycle of Embodiment I is conducted as described above. However, after the daughter nucleic acid sequences are identified, the host cells containing these sequences are retained.

The parent mutated specific nucleic acid population, either as polynucleotides or cloned into the same vector is introduced into the host cells already containing the daughter nucleic acids. Recombination is allowed to occur in the cells and the next generation of recombinants, or granddaughters are selected by the methods described above.

This cycle can be repeated a number of times until the nucleic acid or peptide having the desired characteristics is obtained. It is contemplated that in subsequent

cycles, the population of mutated sequences which are added to the preferred mutants may come from the parental mutants or any subsequent generation.

In an alternative embodiment, the invention provides a method of conducting a "molecular" backcross of the obtained recombinant specific nucleic acid in order to eliminate any neutral mutations. Neutral mutations are those mutations which do not confer onto the nucleic acid or peptide the desired properties. Such mutations may however confer on the nucleic acid or peptide undesirable characteristics. Accordingly, it is desirable to eliminate such neutral mutations. The method of this invention provide a means of doing so.

In this embodiment, after the mutant nucleic acid, having the desired characteristics, is obtained by the methods of the embodiments, the nucleic acid, the vector having the nucleic acid or the host cell containing the vector and nucleic acid is isolated.

The nucleic acid or vector is then introduced into the host cell with a large excess of the wild-type nucleic acid. The nucleic acid of the mutant and the nucleic acid of the wild-type sequence are allowed to recombine. The resulting recombinants are placed under the same selection as the mutant nucleic acid. Only those recombinants which retained the desired characteristics will be selected. Any silent mutations which do not provide the desired characteristics will be lost through recombination with the wild-type DNA. This cycle can be repeated a number of times until all of the silent mutations are eliminated.

Thus the methods of this invention can be used in a molecular backcross to eliminate unnecessary or silent mutations.

Utility

The *in vivo* recombination method of this invention can be performed blindly on a pool of unknown mutants or alleles of a specific polynucleotide or sequence. However, it is not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, t PA, growth hormone, etc. This approach may be used to generate proteins having altered specificity or activity. The approach may also be useful for the generation of mutant nucleic acid sequences, for example, promoter regions, introns, exons, enhancer sequences, 31 untranslated regions or 51 untranslated regions of genes. Thus this approach may be used to generate genes having increased rates of expression. This approach may also be useful in the study of repetitive DNA sequences. Finally, this approach may be useful to mutate ribozymes or aptamers.

Scaffold-like regions separating regions of diversity in proteins may be particularly suitable for the methods of this invention. The conserved scaffold determines the overall folding by self-association, while displaying relatively unrestricted loops that mediate the specific binding. Examples of such scaffolds are the immunoglobulin beta barrel, and the four-helix bundle. The methods of this invention can be used to create scaffold-like proteins with various combinations of mutated sequences for binding.

The equivalents of some standard genetic matings may also be performed by the methods of this invention. For example, a "molecular" backcross can be performed by repeated mixing of the mutant's nucleic acid with the wild-type nucleic acid while selecting for the mutations of interest. As in traditional breeding, this approach can be used to combine phenotypes from different sources into a background of choice. It is useful, for example, for the removal of neutral mutations that affect unselected characteristics (i.e. immunogenicity). Thus it can be useful to determine which mutations in a protein are involved in the enhanced biological activity and which are not.

Peptide Display Methods

The present method can be used to shuffle, by *in vitro* and/or *in vivo* recombination by any of the disclosed methods, and in any combination, polynucleotide sequences selected by peptide display methods, wherein an associated polynucleotide

encodes a displayed peptide which is screened for a phenotype (e.g., for affinity for a predetermined receptor (ligand).

An increasingly important aspect of bio-pharmaceutical drug development and molecular biology is the identification of peptide structures, including the primary amino acid sequences, of peptides or peptidomimetics that interact with biological macromolecules. one method of identifying peptides that possess a desired structure or functional property, such as binding to a predetermined biological macromolecule (e.g., a receptor), involves the screening of a large library or peptides for individual library members which possess the desired structure or functional property conferred by the amino acid sequence of the peptide.

In addition to direct chemical synthesis methods for generating peptide libraries, several recombinant DNA methods also have been reported. One type involves the display of a peptide sequence, antibody, or other protein on the surface of a bacteriophage particle or cell. Generally, in these methods each bacteriophage particle or cell serves as an individual library member displaying a single species of displayed peptide in addition to the natural bacteriophage or cell protein sequences. Each bacteriophage or cell contains the nucleotide sequence information encoding the particular displayed peptide sequence; thus, the displayed peptide sequence can be ascertained by nucleotide sequence determination of an isolated library member.

A well-known peptide display method involves the presentation of a peptide sequence on the surface of a filamentous bacteriophage, typically as a fusion with a bacteriophage coat protein. The bacteriophage library can be incubated with an immobilized, predetermined macromolecule or small molecule (e.g., a receptor) so that bacteriophage particles which present a peptide sequence that binds to the immobilized macromolecule can be differentially partitioned from those that do not present peptide sequences that bind to the predetermined macromolecule. The bacteriophage particles (i.e., library members) which are bound to the immobilized macromolecule are then recovered and replicated to amplify the selected bacteriophage sub-population for a subsequent round of affinity enrichment and phage replication. After several rounds of affinity enrichment and phage replication, the bacteriophage library members that are

thus selected are isolated and the nucleotide sequence encoding the displayed peptide sequence is determined, thereby identifying the sequence(s) of peptides that bind to the predetermined macromolecule (e.g., receptor). Such methods are further described in PCT patent publication Nos. 91/17271, 91/18980, and 91/19818 and 93/08278.

The latter PCT publication describes a recombinant DNA method for the display of peptide ligands that involves the production of a library of fusion proteins with each fusion protein composed of a first polypeptide portion, typically comprising a variable sequence, that is available for potential binding to a predetermined macromolecule, and a second polypeptide portion that binds to DNA, such as the DNA vector encoding the individual fusion protein. When transformed host cells are cultured under conditions that allow for expression of the fusion protein, the fusion protein binds to the DNA vector encoding it. Upon lysis of the host cell, the fusion protein/vector DNA complexes can be screened against a predetermined macromolecule in much the same way as bacteriophage particles are screened in the phage-based display system, with the replication and sequencing of the DNA vectors in the selected fusion protein/vector DNA complexes serving as the basis for identification of the selected library peptide sequence(s).

Other systems for generating libraries of peptides and like polymers have aspects of both the recombinant and *in vitro* chemical synthesis methods. In these hybrid methods, cell-free enzymatic machinery is employed to accomplish the *in vitro* synthesis of the library members (i.e., peptides or polynucleotides). In one type of method, RNA molecules with the ability to bind a predetermined protein or a predetermined dye molecule were selected by alternate rounds of selection and PCR amplification (Tuerk and Gold (1990) Science 249: 505; Ellington and Szostak (1990) Nature 346: 818). A similar technique was used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) Nucleic Acids Res. 18: 3203; Beaudry and Joyce (1992) Science 257: 635; PCT patent publication Nos. 92/05258 and 92/14843). In a similar fashion, the technique of *in vitro* translation has been used to synthesize proteins of interest and has been proposed as a method for generating large libraries of peptides. These methods which rely upon *in vitro* translation, generally

comprising stabilized polysome complexes, are described further in PCT patent publication Nos. 88/08453, 90/05785, 90/07003, 91/02076, 91/05058, and 92/02536. Applicants have described methods in which library members comprise a fusion protein having a first polypeptide portion with DNA binding activity and a second polypeptide portion having the library member unique peptide sequence; such methods are suitable for use in cell-free *in vitro* selection formats, among others.

The displayed peptide sequences can be of varying lengths, typically from 3-5000 amino acids long or longer, frequently from 5-100 amino acids long, and often from about 8-15 amino acids long. A library can comprise library members having varying lengths of displayed peptide sequence, or may comprise library members having a fixed length of displayed peptide sequence. Portions or all of the displayed peptide sequence(s) can be random, pseudorandom, defined set kernal, fixed, or the like. The present display methods include methods for *in vitro* and *in vivo* display of single-chain antibodies, such as nascent scFv on polysomes or scfv displayed on phage, which enable large-scale screening of scfv libraries having broad diversity of variable region sequences and binding specificities.

The present invention also provides random, pseudorandom, and defined sequence framework peptide libraries and methods for generating and screening those libraries to identify useful compounds (e.g., peptides, including single-chain antibodies) that bind to receptor molecules or epitopes of interest or gene products that modify peptides or RNA in a desired fashion. The random, pseudorandom, and defined sequence framework peptides are produced from libraries of peptide library members that comprise displayed peptides or displayed single-chain antibodies attached to a polynucleotide template from which the displayed peptide was synthesized. The mode of attachment may vary according to the specific embodiment of the invention selected, and can include encapsulation in a phage particle or incorporation in a cell.

A method of affinity enrichment allows a very large library of peptides and single-chain antibodies to be screened and the polynucleotide sequence encoding the desired peptide(s) or single-chain antibodies to be selected. The polynucleotide can then be isolated and shuffled to recombine combinatorially the amino acid sequence of the

selected peptide(s) (or predetermined portions thereof) or single-chain antibodies (or just VHI, VLI or CDR portions thereof). Using these methods, one can identify a peptide or single-chain antibody as having a desired binding affinity for a molecule and can exploit the process of shuffling to converge rapidly to a desired high-affinity peptide or scfv. The peptide or antibody can then be synthesized in bulk by conventional means for any suitable use (e.g., as a therapeutic or diagnostic agent).

A significant advantage of the present invention is that no prior information regarding an expected ligand structure is required to isolate peptide ligands or antibodies of interest. The peptide identified can have biological activity, which is meant to include at least specific binding affinity for a selected receptor molecule and, in some instances, will further include the ability to block the binding of other compounds, to stimulate or inhibit metabolic pathways, to act as a signal or messenger, to stimulate or inhibit cellular activity, and the like.

The present invention also provides a method for shuffling a pool of polynucleotide sequences selected by affinity screening a library of polysomes displaying nascent peptides (including single-chain antibodies) for library members which bind to a predetermined receptor (e.g., a mammalian proteinaceous receptor such as, for example, a peptidergic hormone receptor, a cell surface receptor, an intracellular protein which binds to other protein(s) to form intracellular protein complexes such as hetero-dimers and the like) or epitope (e.g., an immobilized protein, glycoprotein, oligosaccha-ride, and the like).

Polynucleotide sequences selected in a first selection round (typically by affinity selection for binding to a receptor (e.g., a ligand)) by any of these methods are pooled and the pool(s) is/are shuffled by *in vitro* and/or *in vivo* recombination to produce a shuffled pool comprising a population of recombined selected polynucleotide sequences. The recombined selected polynucleotide sequences are subjected to at least one subsequent selection round. The polynucleotide sequences selected in the subsequent selection round(s) can be used directly, sequenced, and/or subjected to one or more additional rounds of shuffling and subsequent selection. Selected sequences can also be back-crossed with polynucleotide sequences encoding neutral sequences (i.e.,

having insubstantial functional effect on binding), such as for example by back-crossing with a wild-type or naturally-occurring sequence substantially identical to a selected sequence to produce native-like functional peptides, which may be less immunogenic. Generally, during back-crossing subsequent selection is applied to retain the property of binding to the predetermined receptor (ligand).

Prior to or concomitant with the shuffling of selected sequences, the sequences can be mutagenized. In one embodiment, selected library members are cloned in a prokaryotic vector (e.g., plasmid, phagemid, or bacteriophage) wherein a collection of individual colonies (or plaques) representing discrete library members are produced. Individual selected library members can then be manipulated (e.g., by site-directed mutagenesis, cassette mutagenesis, chemical mutagenesis, PCR mutagenesis, and the like) to generate a collection of library members representing a kernal of sequence diversity based on the sequence of the selected library member. The sequence of an individual selected library member or pool can be manipulated to incorporate random mutation, pseudorandom mutation, defined kernal mutation (i.e., comprising variant and invariant residue positions and/or comprising variant residue positions which can comprise a residue selected from a defined subset of amino acid residues), codon-based mutation, and the like, either segmentally or over the entire length of the individual selected library member sequence. The mutagenized selected library members are then shuffled by *in vitro* and/or *in vivo* recombinatorial shuffling as disclosed herein.

The invention also provides peptide libraries comprising a plurality of individual library members of the invention, wherein (1) each individual library member of said plurality comprises a sequence produced by shuffling of a pool of selected sequences, and (2) each individual library member comprises a variable peptide segment sequence or single-chain antibody segment sequence which is distinct from the variable peptide segment sequences or single-chain antibody sequences of other individual library members in said plurality (although some library members may be present in more than one copy per library due to uneven amplification, stochastic probability, or the like).

The invention also provides a product-by-process, wherein selected polynucleotide sequences having (or encoding a peptide having) a predetermined binding

specificity are formed by the process of: (1) screening a displayed peptide or displayed single-chain antibody library against a predetermined receptor (e.g., ligand) or epitope (e.g., antigen macromolecule) and identifying and/or enriching library members which bind to the predetermined receptor or epitope to produce a pool of selected library members, (2) shuffling by recombination the selected library members (or amplified or cloned copies thereof) which binds the predetermined epitope and has been thereby isolated and/or enriched from the library to generate a shuffled library, and (3) screening the shuffled library against the predetermined receptor (e.g., ligand) or epitope (e.g., antigen macromolecule) and identifying and/or enriching shuffled library members which bind to the predetermined receptor or epitope to produce a pool of selected shuffled library members.

Antibody Display and Screening Methods

The present method can be used to shuffle, by *in vitro* and/or *in vivo* recombination by any of the disclosed methods, and in any combination, polynucleotide sequences selected by antibody display methods, wherein an associated polynucleotide encodes a displayed antibody which is screened for a phenotype (e.g., for affinity for binding a predetermined antigen (ligand).

Various molecular genetic approaches have been devised to capture the vast immunological repertoire represented by the extremely large number of distinct variable regions which can be present in immunoglobulin chains. The naturally-occurring germ line immunoglobulin heavy chain locus is composed of separate tandem arrays of variable segment genes located upstream of a tandem array of diversity segment genes, which are themselves located upstream of a tandem array of joining (i) region genes, which are located upstream of the constant region genes. During B lymphocyte development, V-D-J rearrangement occurs wherein a heavy chain variable region gene (VH) is formed by rearrangement to form a fused D segment followed by rearrangement with a V segment to form a V-D-J joined product gene which, if productively rearranged, encodes a functional variable region (VH) of a heavy chain. Similarly, light chain loci

rearrange one of several V segments with one of several J segments to form a gene encoding the variable region (VL) of a light chain.

The vast repertoire of variable regions possible in immunoglobulins derives in part from the numerous combinatorial possibilities of joining V and i segments (and, in the case of heavy chain loci, D segments) during rearrangement in B cell development. Additional sequence diversity in the heavy chain variable regions arises from non-uniform rearrangements of the D segments during V-D-J joining and from N region addition. Further, antigen-selection of specific B cell clones selects for higher affinity variants having non-germline mutations in one or both of the heavy and light chain variable regions; a phenomenon referred to as "affinity maturation" or "affinity sharpening". Typically, these "affinity sharpening" mutations cluster in specific areas of the variable region, most commonly in the complementarity-determining regions (CDRs).

In order to overcome many of the limitations in producing and identifying high-affinity immunoglobulins through antigen-stimulated ß cell development (i.e., immunization), various prokaryotic expression systems have been developed that can be manipulated to produce combinatorial antibody libraries which may be screened for high-affinity antibodies to specific antigens. Recent advances in the expression of antibodies in Escherichia coli and bacteriophage systems (see, "Alternative Peptide Display Methods", infra) have raised the possibility that virtually any specificity can be obtained by either cloning antibody genes from characterized hybridomas or by de novo selection using antibody gene libraries (e.g., from Ig CDNA).

Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al. (1989) Science 246: 1275; Caton and Koprowski (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 6450; Mullinax et al (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 8095; Persson et al. (1991) Proc. Natl. Acad. Sci. (TJ.S.A.) 88: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4363; Clackson et al. (1991) Nature 352: 624; McCafferty et al. (1990)

Nature 348: 552; Burton et al. (1991) Proc. Natl- Acad. Sci. (U.S.A.) 88: 10134; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133; Chang et al. (1991) J. Immunol. 147: 3610; Breitling et al. (1991) Gene 104: 147; Marks et al. (1991) J. Mol. Biol. 222@: 581; Barbas et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 4457; Hawkins and Winter (1992) J. Immunol. 22: 867; Marks et al. (1992) Biotechnology 10: 779; Marks et al. (1992) J. Biol. Chem. 267: 16007; Lowman et al (1991) Biochemistry 30: 10832; Lerner et al. (1992) Science. 258: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with a receptor (e.g., polypeptide, carbohydrate, glycoprotein, nucleic acid) that is immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

One particularly advantageous approach has been the use of so-called single-chain fragment variable (scfv) libraries (Marks et al. (1992) Biotechnology 10: 779; Winter G and Milstein C (1991) Nature 349: 293; Clackson et al. (1991) op. cit.; Marks et al. (1991) J. Mol. Biol. 222: 581; Chaudhary et al. (1990) Proc. Natl. Acad. Sci. (USA) 87: 1066; Chiswell et al. (1992) TIBTECH 10: 80; McCafferty et al. (1990) op.cit.; and Huston et al- (1988) Proc. Natl. Acad. Sci. (USA) 85: 5879). Various embodiments of scfv libraries displayed on bacteriophage coat proteins have been described.

Beginning in 1988, single-chain analogues of Fv fragments and their fusion proteins have been reliably generated by antibody engineering methods. The first step generally involves obtaining the genes encoding VH and VL domains with desired binding properties; these V genes may be isolated from a specific hybridoma cell line, selected from a combinatorial V-gene library, or made by V gene synthesis. The single-chain Fv is formed by connecting the component V genes with an oligonucleotide that encodes an appropriately designed linker peptide, such as ~~(Gly-Gly-Gly-Gly-Ser)3~~ (Gly-Gly-Gly-Gly-Ser)$_3$ (Seq ID NO: 1) or equivalent linker peptide(s). The linker bridges the C-terminus of the first V region and N-terminus of the second, ordered as either VH-linker-VL or VL-linker-VH' In principle, the scfv binding site can faithfully replicate both the affinity and specificity of its parent antibody combining site.

Thus, scfv fragments are comprised of VH and VL domains linked into a single polypeptide chain by a flexible linker peptide. After the scfv genes are assembled, they are cloned into a phagemid and expressed at the tip of the M13 phage (or similar filamentous bacteriophage) as fusion proteins with the bacteriophage PIII (gene 3) coat protein. Enriching for phage expressing an antibody of interest is accomplished by panning the recombinant phage displaying a population scfv for binding to a predetermined epitope (e.g., target antigen, receptor).

The linked polynucleotide of a library member provides the basis for replication of the library member after a screening or selection procedure, and also provides the basis for the determination, by nucleotide sequencing, of the identity of the displayed peptide sequence or VH and VL amino acid sequence. The displayed peptide (s) or single-chain antibody (e. g., scfv) and/or its VH and VL domains or their CDRs can be cloned and expressed in a suitable expression system. often polynucleotides encoding the isolated VH and VL domains will be ligated to polynucleotides encoding constant regions (CH and CL) to form polynucleotides encoding complete antibodies (e.g., chimeric or fully-human), antibody fragments, and the like. Often polynucleotides encoding the isolated CDRs will be grafted into polynucleotides encoding a suitable variable region framework (and optionally constant regions) to form polynucleotides encoding complete antibodies (e.g., humanized or fully-human), antibody fragments, and the like. Antibodies can be used to isolate preparative quantities of the antigen by immunoaffinity chromatography. Various other uses of such antibodies are to diagnose and/or stage disease (e.g., neoplasia) and for therapeutic application to treat disease, such as for example: neoplasia, autoimmune disease, AIDS, cardiovascular disease, infections, and the like.

Various methods have been reported for increasing the combinatorial diversity of a scfv library to broaden the repertoire of binding species (idiotype spectrum) The use of PCR has permitted the variable regions to be rapidly cloned either from a specific hybridoma source or as a gene library from non-immunized cells, affording combinatorial diversity in the assortment of VH and VL cassettes which can be combined. Furthermore, the VH and VL cassettes can themselves be diversified, such

as by random, pseudorandom, or directed mutagenesis. Typically, VH and VL cassettes are diversified in or near the complementarity-determining regions (CDRS), often the third CDR, CDR3. Enzymatic inverse PCR mutagenesis has been shown to be a simple and reliable method for constructing relatively large libraries of scfv site-directed mutants (Stemmer et al. (1993) Biotechniques 14: 256), as has error-prone PCR and chemical mutagenesis (Deng et al. (1994) J. Biol. Chem. 269: 953 3). Riechmann et al. (1993) Biochemistry 32: 8848 showed semi-rational design of an antibody scfv fragment using site-directed randomization by degenerate oligonucleotide PCR and subsequent phage display of the resultant scfv mutants. Barbas et al. (1992) on.cit. attempted to circumvent the problem of limited repertoire sizes resulting from using biased variable region sequences by randomizing the sequence in a synthetic CDR region of a human tetanus toxoid-binding Fab.

CDR randomization has the potential to create approximately 1 x 10^{20} CDRs for the heavy chain CDR3 alone, and a roughly similar number of variants of the heavy chain CDR1 and CDR2, and light chain CDR1-3 variants. Taken individually or together, the combination possibilities of CDR randomization of heavy and/or light chains requires generating a prohibitive number of bacteriophage clones to produce a clone library representing all possible combinations, the vast majority of which will be non-binding. Generation of such large numbers of primary transformants is not feasible with current transformation technology and bacteriophage display systems. For example, Barbas et al. (1992) op.cit. only generated 5 x 10^{7} transformants, which represents only a tiny fraction of the potential diversity of a library of thoroughly randomized CDRS.

Despite these substantial limitations, bacteriophage. display of scfv have already yielded a variety of useful antibodies and antibody fusion proteins. A bispecific single chain antibody has been shown to mediate efficient tumor cell lysis (Gruber et al. (1994) J. Immunol. 152: 5368). Intracellular expression of an anti-Rev scfv has been shown to inhibit HIV-1 virus replication *in vitro* (Duan et al. (1994) Proc. Natl. Acad. Sci. (USA) 91: 5075), and intracellular expression of an anti-p2lrar, scfv has been shown to inhibit meiotic maturation of Xenopus oocytes (Biocca et al. (1993) Biochem. Bioshys. Res. Commun. 197: 422. Recombinant scfv which can be used to diagnose

HIV infection have also been reported, demonstrating the diagnostic utility of scfv (Lilley et al. (1994) J. Immunol. Meth.. 171: 211). Fusion proteins wherein an scFv is linked to a second polypeptide, such as a toxin or fibrinolytic activator protein, have also been reported (Holvost et al. (1992) Eur. J. Biochess. 210: 945; Nicholls et al. (1993) J. Biol. Chem. 268: 5302).

If it were possible to generate scfv libraries having broader antibody diversity and overcoming many of the limitations of conventional CDR mutagenesis and randomization methods which can cover only a very tiny fraction of the potential sequence combinations, the number and quality of scfv antibodies suitable for therapeutic and diagnostic use could be vastly improved. To address this, the *in vitro* and *in vivo* shuffling methods of the invention are used to recombine CDRs which have been obtained (typically via PCR amplification or cloning) from nucleic acids obtained from selected displayed antibodies. Such displayed antibodies can be displayed on cells, on bacteriophage particles, on polysomes, or any suitable antibody display system wherein the antibody is associated with its encoding nucleic acid(s). In a variation, the CDRs are initially obtained from mRNA (or cDNA) from antibody-producing cells (e.g., plasma cells/splenocytes from an immunized wild-type mouse, a human, or a transgenic mouse capable of making a human antibody as in W092/03918, W093/12227, and W094/25585), including hybridomas derived therefrom.

Polynucleotide sequences selected in a first selection round (typically by affinity selection for displayed antibody binding to an antigen (e.g., a ligand) by any of these methods are pooled and the pool(s) is/are shuffled by *in vitro* and/or *in vivo* recombination, especially shuffling of CDRs (typically shuffling heavy chain CDRs with other heavy chain CDRs and light chain CDRs with other light chain CDRS) to produce a shuffled pool comprising a population of recombined selected polynucleotide sequences. The recombined selected polynucleotide sequences are expressed in a selection format as a displayed antibody and subjected to at least one subsequent selection round. The polynucleotide sequences selected in the subsequent selection round(s) can be used directly, sequenced, and/or subjected to one or more additional rounds of shuffling and subsequent selection until an antibody of the desired binding

affinity is obtained. Selected sequences can also be back-crossed with polynucleotide sequences encoding neutral antibody framework sequences (i.e., having insubstantial functional effect on antigen binding), such as for example by back-crossing with a human variable region framework to produce human-like sequence antibodies. Generally, during back-crossing subsequent selection is applied to retain the property of binding to the predetermined antigen.

Alternatively, or in combination with the noted variations, the valency of the target epitope may be varied to control the average binding affinity of selected scfv library members. The target epitope can be bound to a surface or substrate at varying densities, such as by including a competitor epitope, by dilution, or by other method known to those in the art. A high density (valency) of predetermined epitope can be used to enrich for scfv library members which have relatively low affinity, whereas a low density (valency) can preferentially enrich for higher affinity scfv library members.

For generating diverse variable segments, a collection of synthetic oligonucleotides encoding random, pseudorandom, or a defined sequence kernal set of peptide sequences can be inserted by ligation into a predetermined site (e.g., a CDR). Similarly, the sequence diversity of one or more CDRs of the single-chain antibody cassette(s) can be expanded by mutating the CDR(s) with site-directed mutagenesis, CDR-replacement, and the like. The resultant DNA molecules can be propagated in a host for cloning and amplification prior to shuffling, or can be used directly (i.e., may avoid loss of diversity which may occur upon propagation in a host cell) and the selected library members subsequently shuffled.

Displayed peptide/polynucleotide complexes (library members) which encode a variable segment peptide sequence of interest or a single-chain antibody of interest are selected from the library by an affinity enrichment technique. This is accomplished by means of a immobilized macromolecule or epitope specific for the peptide sequence of interest, such as a receptor, other macromolecule, or other epitope species. Repeating the affinity selection procedure provides an enrichment of library members encoding the desired sequences, which may then be isolated for pooling and shuffling, for sequencing, and/or for further propagation and affinity enrichment.

The library members without the desired specificity are removed by washing. The degree and stringency of washing required will be determined for each peptide sequence or single-chain antibody of interest and the immobilized predetermined macromolecule or epitope. A certain degree of control can be exerted over the binding characteristics of the nascent peptide/DNA complexes recovered by adjusting the conditions of the binding incubation and the subsequent washing. The temperature, pH, ionic strength, divalent cations concentration, and the volume and duration of the washing will select for nascent peptide/DNA complexes within particular ranges of affinity for the immobilized macromolecule. Selection based on slow dissociation rate, which is usually predictive of high affinity, is often the most practical route. This may be done either by continued incubation in the presence of a saturating amount of free predetermined macromolecule, or by increasing the volume, number, and length of the washes. In each case, the rebinding of dissociated nascent peptide/DNA or peptide/RNA complex is prevented, and with increasing time, nascent peptide/DNA or peptide/RNA complexes of higher and higher affinity are recovered.

Additional modifications of the binding and washing procedures may be applied to find peptides with special characteristics. The affinities of some peptides are dependent on ionic strength or cation concentration. This is a useful characteristic for peptides that will be used in affinity purification of various proteins when gentle conditions for removing the protein from the peptides are required.

One variation involves the use of multiple binding targets (multiple epitope species, multiple receptor species), such that a scfv library can be simultaneously screened for a multiplicity of scfv which have different binding specificities. Given that the size of a scfv library often limits the diversity of potential scfv sequences, it is typically desirable to us scfv libraries of as large a size as possible. The time and economic considerations of generating a number of very large polysome scFv-display libraries can become prohibitive. To avoid this substantial problem, multiple predetermined epitope species (receptor species) can be concomitantly screened in a single library, or sequential screening against a number of epitope species can be used. In one variation, multiple target epitope species, each encoded on a separate bead (or subset of

beads), can be mixed and incubated with a polysome-display scfv library under suitable binding conditions. The collection of beads, comprising multiple epitope species, can then be used to isolate, by affinity selection, scfv library members. Generally, subsequent affinity screening rounds can include the same mixture of beads, subsets thereof, or beads containing only one or two individual epitope species. This approach affords efficient screening, and is compatible with laboratory automation, batch processing, and high throughput screening methods.

A variety of techniques can be used in the present invention to diversify a peptide library or single-chain antibody library, or to diversify, prior to or concomitant with shuffling, around variable segment peptides found in early rounds of panning to have sufficient binding activity to the predetermined macromolecule or epitope. In one approach, the positive selected peptide/polynucleotide complexes (those identified in an early round of affinity enrichment) are sequenced to determine the identity of the active peptides. Oligonucleotides are then synthesized based on these active peptide sequences, employing a low level of all bases incorporated at each step to produce slight variations of the primary oligonucleotide sequences. This mixture of (slightly) degenerate oligonucleotides is then cloned into the variable segment sequences at the appropriate locations. This method produces systematic, controlled variations of the starting peptide sequences, which can then be shuffled. It requires, however, that individual positive nascent peptide/polynucleotide complexes be sequenced before mutagenesis, and thus is useful for expanding the diversity of small numbers of recovered complexes and selecting variants having higher binding affinity and/or higher binding specificity. In a variation, mutagenic PCR amplification of positive selected peptide/polynucleotide complexes (especially of the variable region sequences, the amplification products of which are shuffled *in vitro* and/or *in vivo* and one or more additional rounds of screening is done prior to sequencing. The same general approach can be employed with single-chain antibodies in order to expand the diversity and enhance the binding affinity/specificity, typically by diversifying CDRs or adjacent framework regions prior to or concomitant with shuffling. If desired, shuffling reactions can be spiked with mutagenic oligonucleotides capable of *in vitro* recombination with the selected library

members can be included. Thus, mixtures of synthetic oligonucleotides and PCR produced polynucleotides (synthesized by error-prone or high-fidelity methods) can be added to the *in vitro* shuffling mix and be incorporated into resulting shuffled library members (shufflants).

The present invention of shuffling enables the generation of a vast library of CDR-variant single-chain antibodies. One way to generate such antibodies is to insert synthetic CDRs into the single-chain antibody and/or CDR randomization prior to or concomitant with shuffling. The sequences of the synthetic CDR cassettes are selected by referring to known sequence data of human CDR and are selected in the discretion of the practitioner according to the following guidelines: synthetic CDRs will have at least 40 percent positional sequence identity to known CDR sequences, and preferably will have at least 50 to 70 percent positional sequence identity to known CDR sequences. For example, a collection of synthetic CDR sequences can be generated by synthesizing a collection of oligonucleotide sequences on the basis of naturally-occurring human CDR sequences listed in Kabat et al. (199 1) op. cit. ; the pool (s) of synthetic CDR sequences are calculated to encode CDR peptide sequences having at least 40 percent sequence identity to at least one known naturally-occurring human CDR sequence. Alternatively, a collection of naturally-occurring CDR sequences may be compared to generate consensus sequences so that amino acids used at a residue position frequently (i.e., in at least 5 percent of known CDR sequences) are incorporated into the synthetic CDRs at the corresponding position(s). Typically, several (e.g., 3 to about 50) known CDR sequences are compared and observed natural sequence variations between the known CDRs are tabulated, and a collection of oligonucleotides encoding CDR peptide sequences encompassing all or most permutations of the observed natural sequence variations is synthesized. For example but not for limitation, if a collection of human VH CDR sequences have carboxy-terminal amino acids which are either Tyr, Val, Phe, or Asp, then the pool(s) of synthetic CDR oligonucleotide sequences are designed to allow the carboxy-terminal CDR residue to be any of these amino acids. In some embodiments, residues other than those which naturally-occur at a residue position in the collection of CDR sequences are incorporated: conservative amino acid substitutions are

frequently incorporated and up to 5 residue positions may be varied to incorporate non-conservative amino acid substitutions as compared to known naturally-occurring CDR sequences. Such CDR sequences can be used in primary library members (prior to first round screening) and/or can be used to spike *in vitro* shuffling reactions of selected library member sequences. Construction of such pools of defined and/or degenerate sequences will be readily accomplished by those of ordinary skill in the art.

The collection of synthetic CDR sequences comprises at least one member that is not known to be a naturally-occurring CDR sequence. It is within the discretion of the practitioner to include or not include a portion of random or pseudorandom sequence corresponding to N region addition in the heavy chain CDR; the N region sequence ranges from 1 nucleotide to about 4 nucleotides occurring at V-D and D-J junctions. A collection of synthetic heavy chain CDR sequences comprises at least about 100 unique CDR sequences, typically at least about 1,000 unique CDR sequences, preferably at least about 10,000 unique CDR sequences, frequently more than 50,000 unique CDR sequences; however, usually not more than about 1 x 10 6 unique CDR sequences are included in the collection, although occasionally 1 x 107 to 1 *X* 108 unique CDR sequences are present, especially if conservative amino acid substitutions are permitted at positions where the conservative amino acid substituent is not present or is rare (i.e., less than 0.1 percent) in that position in naturally-occurring human CDRS. In general, the number of unique CDR sequences included in a library should not exceed the expected number of primary transformants in the library by more than a factor of 10. Such single-chain antibodies generally bind of about at least 1 x 10 m-, preferably with an affinity of about at least 5 x 10 (superscript 7) M-1, more preferably with an affinity of at least 1 x 10 (superscript 8) M-1 to 1 x 10 (superscript 9) M-1 or more, sometimes up to 1 x 10 (superscript 10) M-1 or more. Frequently, the predetermined antigen is a human protein, such as for example a human cell surface antigen (e. g., CD4, CD8, IL-2 receptor, EGF receptor, PDGF receptor), other human biological macromolecule (e.g., thrombomodulin, protein C, carbohydrate antigen, sialyl Lewis antigen, Lselectin), or nonhuman disease associated macromolecule (e. g., bacterial LPS, virion capsid protein or envelope glycoprotein) and the like.

High affinity single-chain antibodies of the desired specificity can be engineered and expressed in a variety of systems. For example, scfv have been produced in plants (Firek et al. (1993) Plant Mol. Biol. 23: 861) and can be readily made in prokaryotic systems (Owens RJ and Young RJ (1994) J. Immunol. Meth. 168: 149; Johnson S and Bird RE (1991) Methods Enzymol203: 88). Furthermore, the single-chain antibodies can be used as a basis for constructing whole antibodies or various fragments thereof (Kettleborough et al. (1994) Eur. J. Immunol. 24: 952). The variable region encoding sequence may be isolated (e.g., by PCR amplification or subcloning) and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic uses where immunogenicity is preferably minimized. The polynucleotide(s) having the resultant fully human encoding sequence(s) can be expressed in a host cell (e.g., from an expression vector in a mammalian cell) and purified for pharmaceutical formulation.

The DNA expression constructs will typically include an expression control DNA sequence operably linked to the coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the mutant' "engineered" antibodies.

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to an expression control sequence (i.e., positioned to ensure the transcription and translation of the structural gene). These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

In addition to eukaryotic microorganisms such as yeast, mammalian tissue cell culture may also be used to produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, *N.i., N.Y.* (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc, but preferably transformed Bcells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al. (1986) Immunol. Rev. 89: 49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, cytomegalovirus, SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Eukaryotic DNA transcription can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting sequences of between 10 to 300 bp that increase transcription by a promoter. Enhancers can effectively increase transcription when either 51 or 31 to the transcription unit. They are also effective if located within an intron or within the coding sequence itself. Typically, viral enhancers are used, including SV40 enhancers, cytomegalovirus enhancers,, polyoma enhancers, and adenovirus enhancers. Enhancer sequences from mammalian systems are also commonly used, such as the mouse immunoglobulin heavy chain enhancer.

Mammalian expression vector systems will also typically include a selectable marker gene. Examples of suitable markers include, the dihydrofolate reductase gene (DHFR), the thymidine kinase gene (TK), or prokaryotic genes conferring drug resistance. The first two marker genes prefer the use of mutant cell lines that lack the ability to grow without the addition of thymidine to the growth medium. Transformed cells can then be identified by their ability to grow on non-supplemented media. Examples of prokaryotic drug resistance genes useful as markers include genes conferring resistance to G418, mycophenolic acid and hygromycin.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment. lipofection, or electroporation may be used for other cellular hosts. Other methods used to transform mammalian cells include the use of Polybrene, protoplast fusion, liposomes, electroporation, and micro-injection (see, generally, Sambrook et al., supra).

Once expressed, the antibodies, individual mutated immunoglobulin chains, mutated antibody fragments, and other immunoglobulin polypeptides of the invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fraction column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or in developing and performing assay procedures, immunofluorescent stainings, and the like (see, generally, Immunological Methods, Vols. I and II, Eds. Lefkovits and Pernis, Academic Press, New York, N.Y. (1979 and 1981)).

The antibodies generated by the method of the present invention can be used for diagnosis and therapy. By way of illustration and not limitation, they can be used to treat cancer, autoimmune diseases, or viral infections. For treatment of cancer, the antibodies will typically bind to an antigen expressed preferentially on cancer cells, such as erbB-2, CEA, CD33, and many other antigens and binding members well known to those skilled in the art.

Yeast Two-Hybrid Screening Assays

Shuffling can also be used to recombinatorially diversify a pool of selected library members obtained by screening a two-hybrid screening system to identify library members which bind a predetermined polypeptide sequence. The selected library members are pooled and shuffled by *in vitro* and/or *in vivo* recombination. The shuffled pool can then be screened in a yeast two hybrid system to select library members which

bind said predetermined polypeptide sequence (e. g., and SH2 domain) or which bind an alternate predetermined polypeptide sequence (e.g., an SH2 domain from another protein species).

An approach to identifying polypeptide sequences which bind to a predetermined polypeptide sequence has been to use a so-called "two-hybrid" system wherein the predetermined polypeptide sequence is present in a fusion protein (Chien et al. (1991) Proc. Natl. Acad. Sci. (USA) 88: 9578). This approach identifies protein-protein interactions *in vivo* through reconstitution of a transcriptional activator (Fields S and Song 0 (1989) Nature 340: 245), the yeast Gal4 transcription protein. Typically, the method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Polynucleotides encoding two hybrid proteins, one consisting of the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a known protein and the other consisting of the Gal4 activation domain fused to a polypeptide sequence of a second protein, are constructed and introduced into a yeast host cell. Intermolecular binding between the two fusion proteins reconstitutes the Gal4 DNA-binding domain with the Gal4 activation domain, which leads to the transcriptional activation of a reporter gene (e.g., *lacz, HIS3)* which is operably linked to a Gal4 binding site. Typically, the two-hybrid method is used to identify novel polypeptide sequences which interact with a known protein (Silver SC and Hunt SW (1993) Mol. Biol. Rep. 17: 155; Durfee et al. (1993) Genes Devel. 7; 555; Yang et al. (1992) Science 257: 680; Luban et al. (1993) Cell 73: 1067; Hardy et al(1992) Genes Devel. 6; 801; Bartel et al. (1993) Biotechniques 14: 920; and Vojtek et al. (1993) Cell 74: 205). However, variations of the two-hybrid method have been used to identify mutations of a known protein that affect its binding to a second known protein (Li B and Fields S (1993) FASEB J. 7: 957; Lalo et al. (1993) Proc. Natl. Acad. Sci. (USA) 90: 5524; Jackson et al(1993) Mol. Cell. Biol. 13; 2899; and Madura et al. (1993) J. Biol- Chem. 268: 12046). Two-hybrid systems have also been used to identify interacting structural domains of two known proteins (Bardwell et al. (1993) med. Microbial. 8: 1177; Chakrabarty et al. (1992) J. Biol. Chem. 267: 17498; Staudinger et al. (1993) J. Biol. Chem. 268: 4608; and Milne GT.

and Weaver DT (1993) Genes Devel. 7; 1755) or domains responsible for oligomerization of a single protein (Iwabuchi et al. (1993) Oncogene 8; 1693; Bogerd et al. (1993) J. Virol. 67: 5030). Variations of two-hybrid systems have been used to study the *in vivo* activity of a proteolytic enzyme (Dasmahapatra et al. (1992) Proc. Natl. Acad.. Sci. (USA) 89: 4159). Alternatively, an E. coli/BCCP interactive screening system (Germino et al. (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 933; Guarente L (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 1639) can be used to identify interacting protein sequences (i.e., protein sequences which heterodimerize or form higher order heteromultimers). Sequences selected by a two-hybrid system can be pooled and shuffled and introduced into a two-hybrid system for one or more subsequent rounds of screening to identify polypeptide sequences which bind to the hybrid containing the predetermined binding sequence. The sequences thus identified can be compared to identify consensus sequence(s) and consensus sequence kernals.

In general, standard techniques of recombination DNA technology are described in various publications, e.g. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory; Ausubel et al., 1987, Current Protocols in Molecular Biology, vols. 1 and 2 and supplements, and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Technicrues (1987), Academic Press, Inc., San Diego, CA, each of which is incorporated herein in their entirety by reference. Polynucleotide modifying enzymes were used according to the manufacturers recommendations. Oligonucleotides were synthesized on an Applied Biosystems Inc. Model 394 DNA synthesizer using ABI chemicals. If desired, PCR amplimers for amplifying a predetermined DNA sequence may be selected at the discretion of the practitioner.

The following non-limiting examples are provided to illustrate the present invention.

Example 1

Generation of Random Size Polynucleotides Using U.V. Induced Photoproducts

One microgram samples of template DNA are obtained and treated with U.V. light to cause the formation of dimers, including TT dimers, particularly purine dimers. U.V. exposure is limited so that only a few photoproducts are generated per gene on the template DNA sample. Multiple samples are treated with U.V. light for varying periods of time to obtain template DNA samples with varying numbers of dimers from U.V. exposure.

A random priming kit which utilizes a non-proofreading polymease (for example, ~~Prime-It~~ PRIME-IT® II Random Primer Labeling kit by ~~Stratagene~~ STRATAGENE™ Corporation Cloning Systems) is utilized to generate different size polynucleotides by priming at random sites on templates which are prepared by U.V. light (as described above) and extending along the templates. The priming protocols such as described in the ~~Prime-It~~ PRIME-IT® II Random Primer Labeling kit may be utilized to extend the primers. The dimers formed by U.V. exposure serve as a roadblock for the extension by the non-proofreading polymerase. Thus, a pool of random size polynucleotides is present after extension with the random primers is finished.

Example 2

Isolation of Random Size Polynucleotides

Polynucleotides of interest which are generated according to Example 1 are are gel isolated on a 1.5% agarose gel. Polynucleotides in the 100-300 bp range are cut out of the gel and 3 volumes of 6 M NaI is added to the gel slice. The mixture is incubated at 50 °C for 10 minutes and 10 µl of glass milk (Bio 101) is added. The mixture is spun for 1 minute and the supernatant is decanted. The pellet is washed with 500 µl of Column Wash (Column Wash is 50% ethanol, 10mM Tris-HCl pH 7.5, 100 mM NaCl and 2.5 mM EDTA) and spin for 1 minute, after which the supernatant is decanted. The washing, spinning and decanting steps are then repeated. The glass milk pellet is resuspended in 20µl of H_2O and spun for 1 minute. DNA remains in the aqueous phase.

Example 3

Shuffling of Isolated Random Size 100-300bp Polynucleotides

The 100-300 bp polynucleotides obtained in Example 2 are recombined in an annealing mixture (0.2 mM each dNTP, 2.2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl ph 8.8, 0.1% ~~Triton X-100~~ TRITON X-100® detergent, 0.3 μ; ~~Taq~~ TAQ® DNA polymerase, 50 μl total volume) without adding primers. A ~~Robocycler~~ ROBOCYCLER® thermal cycler by ~~Stratagene~~ STRATAGENE™ Corporation was used for the annealing step with the following program: 95 °C for 30 seconds, 25-50 cycles of [95 °C for 30 seconds, 50 - 60 °C (preferably 58 °C) for 30 seconds, and 72 °C for 30 seconds] and 5 minutes at 72 °C. Thus, the 100-300 bp polynucleotides combine to yield double-stranded polynucleotides having a longer sequence. After separating out the reassembled double-stranded polynucleotides and denaturing them to form single stranded polynucleotides, the cycling is optionally again repeated with some samples utilizing the single strands as template and primer DNA and other samples utilizing random primers in addition to the single strands.

Example 4

Screening of Polypeptides from Shuffled Polynucleotides

The polynucleotides of Example 3 are separated and polypeptides are expressed therefrom. The original template DNA is utilized as a comparative control by obtaining comparative polypeptides therefrom. The polypeptides obtained from the shuffled polynucleotides of Example 3 are screened for the activity of the polypeptides obtained from the original template and compared with the activity levels of the control. The shuffled polynucleotides coding for interesting polypeptides discovered during screening are compared further for secondary desirable traits. Some shuffled polynucleotides corresponding to less interesting screened polypeptides are subjected to reshuffling.

As can be appreciated from the above description, the present invention has a wide variety of applications. Variations without departing from the scope and intention of the present invention will be readily apparent to one of ordinary skill upon reviewing

the above. Such variations are expected to be within the ordinary skill of the average practitioner and are encompassed by the present invention.